PRODUCTION OF INCOMPLETE INFLUENZA VIRUS IN THE DEEMBRYONATED EGG

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In the course of studies on double neutralization of virus produced from cells doubly infected with WS and MEL strains of influenza virus (Burnet and Lind, 1954b), it was observed that continued passage of high concentrations of virus in the deembryonated egg soon became impossible owing to increasing incompleteness of the virus. This suggested to us that the deembryonated egg might be a very suitable system for the study of the nature of incomplete virus. In particular, it was soon realised that the technique could be used to provide a new means of measuring the infectivity of a given influenza virus preparation, i.e., by measuring the amount of haemagglutinin produced in a standard time from the first cycle of infection in the deembryonated egg with inocula of known haemagglutinin content. Comparison of such titrations with those made by conventional methods showed gross systematic discrepancies which are interpreted as showing that in a typical "incomplete" virus preparation there are many particles which can initiate infection in the sense of inducing the formation and liberation of haemagglutinin from the cells invaded, but which cannot give rise to the continuing chain of infections needed to produce a detectable haemagglutinin titre from a very small inoculum. This introduces a new concept into the already complex subject of the nature of intracellular multiplication of influenza virus.

A second virtue of the same technical approach is in regard to the demonstration of interference by incomplete virus. For this, the first cycle harvest is removed at 7 hours and replaced by new medium. The amount of virus liberated in the ensuing 15 hours will be determined by several factors including particularly interference by non-replicating virus. With incomplete virus the curves of the relation of virus yield to inoculum may show a well defined minimum at which is assumed to be the virus dose providing one functioning virus particle (infective or interfering) per cell.

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MATERIALS AND METHODS.

Virus strains.

Most of the work was done with strain WS–, obtained by recombination between the influenza A strains MEL and WSE (Burnet and Lind, 1952). This strain was chosen primarily for its convenience in recombination experiments, one of the main objectives of the whole series of experiments being to obtain genetic evidence of the nature of incompleteness. The reciprocal recombinant M+ was also used in some experiments.

Diluents.

Saline: 9·0 gm. NaCl made up to 1000 ml. with distilled water.

Ringer's solution. 8·5 gm. NaCl, 0·25 gm. KCl, 0·3 gm. $CaCl_2$ made up to 1000 ml. with distilled water.

G.P.S.R.: Ringer's solution to which 0·1 p.c. glucose, calcium penicillin (12 units/ml.) and dihydrostreptomycin (50 μg./ml.) had been added.

Titration of haemagglutinin (H.A.).

Serial twofold dilutions of virus were prepared in 0·25 ml. volumes in saline and 0·25 ml. of 1 p.c. suspension of fowl red-blood cells was added to each tube. The standard '+' pattern of cell agglutination was taken as endpoint and was read after the cells had settled for 45 minutes at room temperature. Where necessary, it was determined by interpolation. The reciprocal of the end point dilution was taken as titre. It should be noted that the HA titre is used throughout as a measure of concentration, not of absolute amount of virus.

Determination of ID_{50}.

Serial tenfold dilutions of virus fluids were prepared in ice-cold saline containing 10 p.c. normal horse serum, and 0·05 ml. of each dilution was inoculated allantoically into each of 4 11-day-old chick embryos. After 3 days' incubation at 35° C., the eggs were tested for the presence of virus haemagglutinin in the allantoic fluid. ID_{50} end points were calculated by the method of Reed and Muench (1938) and are expressed in terms of ID_{50} per 0·05 ml. of the original fluid.

Determination of mouse infectivity.

Virus fluids were diluted to a HA titre of 1 AD and from this three further tenfold dilutions were prepared, giving 10^0, 10^{-1}, 10^{-2} and 10^{-3} AD. Volumes of 0·05 ml. of each dilution were inoculated intranasally in anaesthetized mice 4–5 weeks old, 4 mice being used per dilution. Deaths were recorded, and at the end of 7 days all surviving mice were killed and examined for lung lesions which were scored conventionally, 0–3·9, according to the degree of consolidation of the lungs. Death on the 5th day or earlier scored 5, on the 6th day 4·5, and on the 7th day 4. The score for any group was expressed as the average lesion score of the 4 mice.

Growth of virus in deembryonated eggs (DEE).

The method first described by Bernkopf (1949) and modified by Burnet and Lind (1954b) was followed. Embryonated eggs at the 13th to 16th day of incubation were opened by drilling away the shell covering the airspace and the entire contents except the chorio-allantoic, allantoic and amniotic membranes were removed. After thorough washing with warm Ringer's solution, each egg received the inoculum in a volume of 5 ml. The eggs were capped and placed on a revolving disc set at an angle of 15° in an incubator maintained at 37° C. with a humidity of 60 p.c. After one hour the inoculum was removed and retained for titration of residual haemagglutinin. In earlier experiments RDE (200 units/ml.) was added to the eggs for one hour at this stage to remove any virus adherent to the surface of

the cells and to inactivate any virus receptors which remained intact, thus preventing virus entering fresh cells after the first growth cycle. However, the finding that the addition of RDE made little difference to the HA or ID_{50} titres of harvests from DEE led us to abandon its use in later experiments. For these, the eggs were washed three times with 10 ml. of warm Ringer solution as soon as the period for virus adsorption had expired, refilled with 5 ml. of warm G.P.S.R. and returned to the incubator. The first harvest was made 7 hours after infection (7-hour fluids) and the eggs were refilled with 5 ml. G.P.S.R. without washing and incubated overnight, when a second harvest was made (22-hour fluids).

Preparation of inocula for DEE experiments.

Freshly-harvested virus from an allantoic passage at a dilution of 10^{-4} of the stock virus was diluted, usually in 0·5 $\log_{10}$ steps, in G.P.S.R. to give fluids with HA titres of 20, 6·3, 2·0, 0·6, 0·2 and 0·06 agglutinating doses (AD). Each dilution was inoculated in a volume of 5 ml. into each of 2 DEE. To initiate the next passage, a pool of the 1-hour harvests from the eggs which received the 20 AD dose in the previous run was diluted to give the same range of doses which were then inoculated into fresh DEE in the same way. As the number of passages progressed and the HA titres of the 7-hour harvest fell, it was necessary to inoculate up to 6 eggs with the 20 AD dose in order to have sufficient virus in the 7-hour harvest to inoculate eggs for the next passage.

In order to clarify the quantitative relationships, we should state that with the techniques used in this laboratory a standard fluid which gives a HA titre of X will give an infectivity end point (ID_{50}) of about $X \times 10^{6 \cdot 6}$, using an inoculum of 0·05 ml. The standard 5 ml. inoculum of virus with HA level X will contain $100X \times 10^{6 \cdot 6}$ ID_{50} since its volume is 100 times that of the standard egg inoculum.

EXPERIMENTAL.

Experiments to show the development of incompleteness with WS—.

Three series of consecutive passages of WS— at an inoculum level of 20 ADs have been made. In two, a full range of doses with HA levels ($\log_{10}$) of 1·3, 0·8, 0·3, $\bar{1}$·8, $\bar{1}$·3, $\bar{2}$·8 was used in each passage, the transfers being made from the 7-hour fluid pooled from all eggs inoculated with 20 ADs of virus. In one only the 20 AD inoculum was used up to the 4th passage. For the 5th passage the whole series of inocula was used.

TABLE 1.

Character of 7-hour fluids obtained on serial DEE passage of WS- in three experiments.

Passage	Expt. 1		Expt. 2		Expt. 3	
	HA	ID_{50}/HA	HA	ID_{50}/HA	HA	ID_{50}/HA
Stock	2·9	4·8	2·8	5·4	2·9	5·6
1	2·1	4·6	2·1	3·7	2·1	3·2
2	2·0	2·1	2·0	3·7	2·3	2·0
3	1·6	0·9	1·9	2·9	1·7	2·1
4	1·3	0·9	1·8	2·0		
5	0·6	1·6	1·3	1·9		

All values are shown in $\log_{10}$ units. "Stock" gives the results of titrating the initial allantoic fluid. Other values are for 7-hour fluids of the DEE passage shown.

In all experiments the amount of virus remaining in the larger inocula after the hour allowed for virus absorption was determined by HA titration. In most cases approximately half the inoculated virus was taken up and, in preparing the graphs, it was regarded as more convenient and devoid of significant error to assume that in all cases half the virus was taken up by the cells. The effective inocula would then be in ADs ($\log_{10}$) 1·0, 0·5, 0·0, $\bar{1}$·5, $\bar{1}$·0, $\bar{2}$·5, and these values are shown on the graphs.

Table 1 shows the course of three experiments. The HA titre of the 7-hour fluids from a 20 AD inoculum is given to indicate the gradual fall in haemagglutinin production and alongside the ratio ID_{50}/HA.

In our hands this value with active virus lies around 5·6 and the degree of incompleteness can be taken as corresponding to the difference between 5·6 and the observed value.

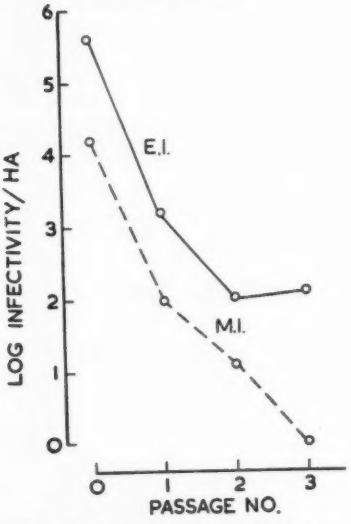

Fig. 1. Fall in the infectivity/HA ratio of WS— on passage as tested by infectivity for the chick embryo and by intranasal inoculation in mice.

Abscissae: Number of DEE passage (Experiment 3).

Ordinates: Logarithmic ratio of infectivity to haemagglutinin titre. Continuous line: Infectivity in terms of $SEID_{50}$ in allantoic cavity. Broken line: Infectivity in terms of mouse-lung pathogenicity, the end point being average grade 2 consolidation at 7 days.

In Experiment 3 the 7-hour fluids of each passage were also titrated in mice to determine their degree of incompleteness by this criterion. The results are compared with those obtained by the conventional method of allantoic inoculation in Fig. 1. This shows a parallel fall for the first two passages, but a significantly greater fall in mouse virulence than in egg infectivity on the 3rd passage. The results would indicate that only chick-embryo-infective particles can produce mouse-lung adhesions and that sometimes the mouse-lung virulence may fall below that corresponding to the egg infectivity.

The most important feature of the experiments was the much greater relative capacity for incomplete fluids to produce haemagglutinin in the DEE than to cause infection in the allantoic cavity with standard infectivity titrations. This is clearly shown by plotting the results of DEE experiments with the haemagglutinin yield at 7 hours as ordinates and the size of the inocula as abscissae. The inoculum can be stated (a) in terms of its HA content, (b) in terms of its infectivity, the distance between the two being a measure of the degree of incompleteness. It will be

seen at once from Figs. 2 and 3 that, although the 7-hour yield per HA unit of inoculum falls progressively with passage, yet the yield per ID_{50} increases to a maximum after the second or third passage. Since the curves are of approxi-

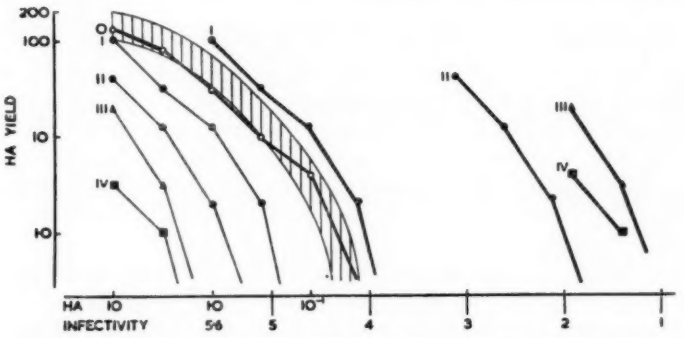

Fig. 2. Haemagglutinin production with successive passage of WS—in DEE. Experiment 1.

Ordinates: HA yield at 7 hours.

Abscissae: Effective concentration of inoculum in terms of HA or infectivity.

Shaded band shows the zone in which lie results from stock (complete) virus, line O being control fluid of this experiment. For stock virus the ratio of log ID_{50}/HA is taken as 5·6, and the single line corresponds to inocula estimated by either method. For each of the subsequent passages there are two lines, single thin line to the left where inoculum is measured by HA, the parallel double (thick) line to the right in terms of infectivity. The distance between the two is a measure of the incompleteness of the virus.

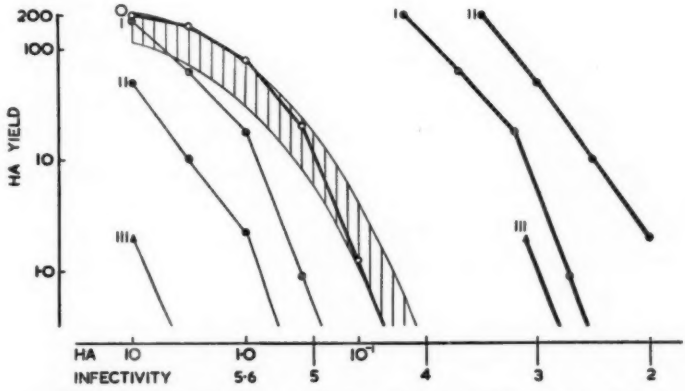

Fig. 3. Haemagglutinin production with successive passage of WS—in DEE. Experiment 3.

Ordinates and abscissae as in Fig. 2.

7-hour yields for comparison with Figs. 1 and 4.

mately the same slope as is found with complete virus, it is virtually impossible that multiplicity reactivation can be wholly responsible for this effect. The evidence for our contention that replication of non-infectious virus takes place to produce HA will be discussed later.

Interference as indicated by the 22-hour yields of haemagglutinin.

When active virus is diluted serially and grown according to our standard procedure in DEEs, the HA titres obtained at 22 hours fall between 200 and 320 until near the end point of infection. In our hands no extension of the DEE infectivity end points was observed by prolonging incubation beyond 22 hours. In almost all experiments the 22-hour HA titres were determined and a number of interesting results were obtained which throw some light on the nature of interference.

It was found with almost complete regularity that when incomplete WS— fluids were titrated the haemagglutinin yield at 22 hours gave a minimum value at approximately the point corresponding to the absorption of 1 AD. Fig. 4 shows the results with control material and the three successive DEE passages of WS— in Experiment 3.

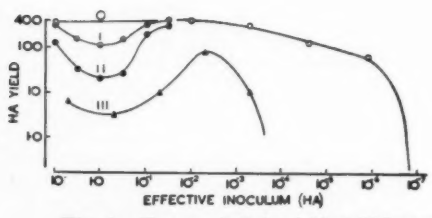

Fig. 4. Haemagglutinin yield at 22 hours from successive passage of WS— in DEE. Experiment 3.

Ordinates: Haemagglutinin yield at 22 hours from DEE emptied and refilled 7 hours after inoculation.

Abscissae: Effective inoculum in terms of HA units.

Roman numerals indicate the successive passages.

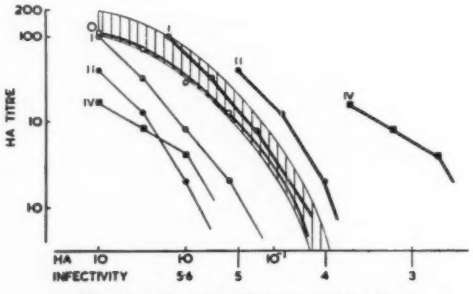

Fig. 5. Haemagglutinin production at 7 hours with successive passage of M+ in DEE.

Ordinates and Abscissae, symbols, etc., as Figs. 2 and 3.

Experiments with M+ and other strains.

Similar experiments with the reciprocal recombinant type M+ were also carried out, using precisely similar methods. Fig. 5 shows the 7-hour yields of haemagglutinin in successive passages in terms both of the HA titre and of the infectivity titre of the fluids used as inocula. It will be seen that until the

4th passage is reached the degree of incompleteness, and hence the evidence for the production of haemagglutinin by non-infective virus, is much less striking than with WS—.

It is convenient to add here brief notes about other strains tested in the same way. These were CAM (Influenza A) and its mouse-adapted variant, CAM-MP, and the standard Influenza B strain LEE. None of these showed any striking development of incompleteness before the experiment had to be terminated by failure of yield. The essential data are combined in Table 2.

TABLE 2.

Character of 7-hour Harvests from Successive DEE Passage Using 20 AD Inoculum.

Passage	M+		CAM		CAM-MP		LEE	
	HA	ID_{50}/HA	HA	ID_{50}/HA	HA	ID_{50}/HA	HA	ID_{50}/HA
Stock	2·8	4·9	2·9	5·4	3·0	5·3	2·5	4·8
1	2·0	4·8	1·8	5·1	2·0	4·3	1·4	4·9
2	2·0	4·0	1·1	4·6	1·8	4·5	0·5	4·3
3	1·6	3·2	0·8	4·7	1·8	3·5		
4	1·3	2·7			1·0	3·7		

Graphs are not shown because, in all, the yield of HA was not significantly different from that of stock virus in terms of infectivity. In other words, there is no evidence from these three strains that non-infective but haemagglutinin-producing virus exists. With CAM-MP there is a distinct suggestion of the effect, but it is much less than is seen with M+, and quite insignificant in comparison with WS—. We have not studied PR8, but from the work of von Magnus, Horsfall, Fazekas and others it appears evident that WS— behaves very similarly, and it is highly probable that our essential findings with WS— could be duplicated with PR8.

Experiments with heated virus and with mixtures of heat-inactivated and active virus.

In several experiments WS— virus, in the form of allantoic fluid buffered with phosphates to pH 7·9, was heated (sealed in small ampoules) at 56° C. for short periods, usually 5 minutes, to reduce the infectivity to an amount comparable to that of an average incomplete virus. Such partially heat-inactivated virus was then tested in DEEs in the same fashion as the virus of the passage series. Preliminary experiments indicated the probability that something equivalent to multiplicity reactivation was occurring and that the interests of the reaction was limited to the larger virus inocula.

Fig. 6 shows the results of the two more detailed experiments done with the same batch of heated virus WS—. This had been diluted 1:5 in 0·1 M

phosphate for heating and had $\log_{10}$ titres of HA, 2·4; ID_{50}, 5·1. The 7-hour and 22-hour yields of haemagglutinin are shown for inocula ranging from HA levels of 50 to 1. By using more dilute cells and large volumes of fluid it was possible to detect down to 1/20 of a HA unit. It will be seen that haemagglutinin production occurs only at what we have taken as a nominal multiplicity of 5 (10 AD original inoculum) or higher, that the slope of the curve is steeper than that observed with incomplete virus, and that the production of haemagglutinin is greater than would be expected from its infectivity.

The 22-hour yields show quite striking evidence of interference produced by the larger inocula, but becoming insignificant below the nominal multiplicity of 2.

Other experiments were made with mixtures of active virus and virus preheated to 56° C. for 10 minutes with a $\log_{10}$ ID_{50}/HA value of approximately 1·0. These showed that the 7-hour results were determined simply by the amount of active virus present in the mixtures, no significant difference from controls being detected. The 22-hour results showed a consistent depression in the yield from the two highest inocula (10 and 3·2 ADs absorbed), the amount of depression increasing with the proportion of inactive virus.

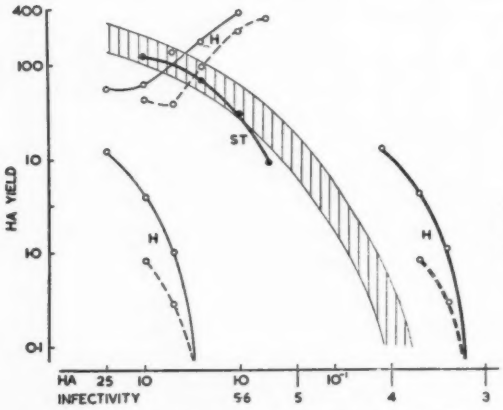

Fig. 6. Haemagglutinin yield from virus WS— partly inactivated by heat.

Two experiments with the same heated material H are shown. The lower curves as in Figs. 2 and 3 show 7-hour yields with the effective concentration of the inocula expressed in terms of haemagglutinin (thin lines to left) or infectivity (double-thick lines to right). Upper curves marked H show the 22-hour yields of haemagglutinin. Continuous and broken lines, results of the two experiments.

ST: Curve showing 7-hour yields of haemagglutinin from eggs inoculated with unheated virus from the same WS— stock.

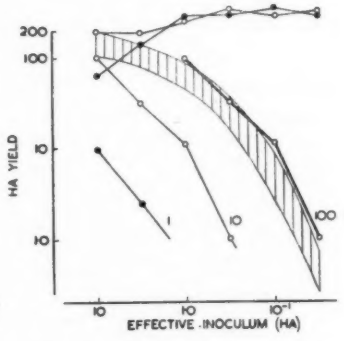

Fig. 7. Behaviour of mixtures of active and heat-inactivated virus.

Ordinates and abscissae as in Figs. 2 and 3.

The three curves labelled 100, 10 and 1 represent the 7-hour yields from mixtures containing 100, 10 and 1 p.c. of active WS— virus in heat-inactivated virus of the same batch. The corresponding 22-hour yields of haemagglutinin for the 10 p.c. and 1 p.c. mixtures are shown above.

The important point is the contrast from the results with incomplete virus in which depression of yield is seen at the middle range of inocula around 1 AD.

Recombination experiments between complete and incomplete viruses.

Recombination experiments were carried out using the M+ and WS— strains of virus. The 7-hour pool of virus, which had undergone one or more DEE passages, was diluted to a HA titre of 10 and mixed in equal quantities with a similar dilution of freshly harvested "complete" virus from the other strain. Two DEE were each doubly infected with 5 ml. of this mixture. The 7-hour harvests from the doubly-infected eggs were titrated for HA, using as diluents saline and a 1/200 dilution of each of the two specific antisera in order to determine whether "anomalous neutralization" occurred (Burnet and Lind, 1954a). It is found that when first growth cycle fluids are titrated in these three diluents, the sum of the HA titre obtained in the two antisera is much less than 50 p.c. of that of the control. This contrasts with the behaviour of an artificial mixture of the viruses concerned when the sum of the HA titres in serum diluents is 50 p.c. or more of that of the saline control. In our experience, "anomalous neutralization" in a first cycle fluid can usually be taken as a sign that recombinant virus strains are present.

The two recombinant strains which result from double infection with M+ and WS— are MEL and WSE (Lind and Burnet, 1953). To determine whether MEL were present, serial tenfold dilutions of the 7-hour first growth cycle fluid were prepared in a 1/25 dilution of WS antiserum and 0·05 ml. volumes of each dilution were inoculated allantoically into whole eggs. After 3 days' incubation at 35° C., all allantoic fluids showing virus haemagglutinin were harvested and submitted to the *in vitro* tests which distinguish MEL from M+ (Burnet and Lind, 1952; Lind and Burnet, 1953).

To demonstrate the presence of WSE, similar tenfold dilutions of the 7-hour harvest were prepared using anti-MEL 1/25 serum as diluent. These dilutions were inoculated on to the chorioallantois of 12-day chick embryos. After 4 days' incubation the eggs were opened and the embryos examined for death and the haemorrhagic lesions typical of WSE infection. Lungs and brains from embryos with lesions were ground and suspended in saline, and, if the haemagglutinin titre were high enough, the suspensions were subjected to the necessary *in vitro* tests which distinguish WSE.

Representative virus preparations which showed the characteristic *in vitro* reactions of MEL or WSE, especially those obtained at the highest dilutions, were taken through a further limit dilution (LD) allantoic passage to confirm their identity.

Table 3 summarizes the results obtained. It will be seen that when incomplete virus replaces active virus as one constituent of the standard inoculum, the

TABLE 3.

Isolation of Recombinants from 7 Hour Fluids from DEE Doubly Infected with Complete and Incomplete Virus Strains.

Experiment I.					Experiment II.				
Parent viruses	Anomalous neutralization		Highest dilution from which recombinant isolated on LD passage[3]		Parent viruses	Anomalous neutralization		Highest dilution from which recombinant isolated on LD passage	
	7 hr. fluid	22 hr. fluid	MEL[1]	WSE[2]		7 hr. fluid	22 hr. fluid	MEL[1]	
M+complete	4				M+complete				
+WS−complete	+(14%)	+(22%)	10^{-5}	10^{-3}	+WS−complete	+(11%)	+(20%)	10^{-6}	
+WS−1st pass	+(13%)	+(17%)	10^{-4}	nil	+WS−1st pass	+(15%)	+(24%)	10^{-5}	
+WS−2nd pass	++(8%)	+(20%)	10^{-3}	10^{-1}	+WS−2nd pass	+(25%)	−(57%)	10^{-4}	
+WS−3rd pass	+(16%)	+(26%)	10^{-4}	10^{-2}	+WS−3rd pass	+(37%)	−(62%)	nil	
+WS−4th pass	++(5%)	+(25%)	10^{-5}	nil	+WS−4th pass	+(18%)	+(20%)	10^{-5}	
WS−complete					WS−complete				
+M+complete	+(30%)	+(36%)	10^{-6}	—	+M+complete	+(11%)	+(20%)	10^{-6}	
+M+1st pass	+(25%)	+(37%)	10^{-5}	10^{-3}	+M+1st pass	+(11%)	+(18%)	10^{-6}	
+M+2nd pass	+(25%)	+(22%)	10^{-5}	10^{-1}	+M+2nd pass	+(16%)	±(45%)	10^{-3}	
+M+3rd pass	+(40%)	+(25%)	10^{-4}	—	+M+3rd pass	+(40%)	+(34%)	10^{-3}	
+M+4th pass	+(13%)	+(26%)	10^{-5}	—	+M+4th pass	+(25%)	+(28%)	10^{-4}	

1. Allantoic titration using anti WS serum 1/25 as diluent.
2. Chorioallantoic titration using anti MEL serum 1/25 as diluent.
3. 7 hour harvest used for LD titration.
4. 14% indicates that the sum of the titres obtained in haemagglutinin titrations in which 1/200 dilutions of each antiserum were used as diluents was 14% of the haemagglutinin titre obtained in saline.

yield of recombinants is virtually the same. The higher yield of MEL than of WSE may be due to interference with the latter by WS— present in the first cycle fluids.

DISCUSSION.

The most important feature of these results is the demonstration that incomplete virus of the strain WS— can produce haemagglutinin to a far higher level than corresponds to its infectivity as judged by the conventional methods of titration in chick embryos and mice. This finding, which has already been briefly reported (Burnet, Lind and Stevens, 1954), makes it necessary to assume the existence of intermediate grades of incompleteness, a concept by no means easy to analyze. We have already indicated that the alternative explanation in terms of multiplicity reactivations is not in accord with the shape of the curves of haemagglutinin production, but some further discussion of the point is desirable before discarding the possibility.

There is active current discussion on the relation between (a) the ID_{50} and the number of particles visible in electron-micrographs and (b) the relation between HA and infectivity titres. Horsfall (1954) believes that, if allantoic fluid virus is obtained under circumstances that allow no opportunity for thermal degradation after its production, every particle is infective for allantoic cells of the 10-day chick embryo. Each particle also has a standard effect on red cells and by a suitably elaborated method of HA titration a direct measure of the number of virus particles per ml. can be obtained. Under the ordinary conditions used for the preparation of standard virus, growth for two days from a small inoculum, only one in four particles can produce infection. Donald and Isaacs (1954) find, by quantitative electron-microscopy, that standard virus contains about 10 visible particles per ID_{50}. The value obtained in an infectivity titration depends, of course, on the nature of the "indicator" being used. Influenza viruses can be very conveniently titrated by Fulton and Armitage's method (1951) of using fragments of chorioallantoic membrane in small containers. The values obtained are reproducible and positive results at the end point are distributed in the fashion to be expected if infection is initiated by single particles. The titre obtained for a given fluid is, however, only one-fortieth of that given by titration in the intact embryo. The deembryonated egg will presumably have a susceptibility as test organism somewhat intermediate between the intact embryo and the tissue fragment.

The relation between the ID_{50} and the HA titre, as determined by the methods in current use in any given laboratory, is one to be determined empirically. In our hands, fresh standard virus from 44 hours' incubation at 35° C. has an ID_{50}/HA ratio of $10^{5.6-5.8}$ for well adapted strains. Making due allowance for the volume used in infectivity titrations and the theoretical relation

between ID_{50} and number of infective particles, then, depending on whether we adopt Horsfall's or Donald and Isaac's figures, the number of virus particles per ml. for a standard fluid of HA titre, X, will be approximately

$$X \times 10^{7.3} \text{ or } X \times 10^{7.7}.$$

There is also a difference of opinion on the number of cells accessible to infection in the allantoic cavity, Fazekas and Cairns (1952) claiming 10^8 while Horsfall estimated $10^{7.3}$. This five-fold difference taken on top of another considerable range of doubt as to the number of virus particles in a preparation of known HA titre makes it impossible to say *a priori* what multiplicity of infection exists in our experimental systems. Without this knowledge, discussion of any hypothesis of multiplicity reactivation must be difficult.

We have, therefore, decided to make an empirical approach and calculate the multiplicity of infection on the assumption that each infected cell, i.e., each cell that is effectively stimulated to produce and liberate virus, will produce the same amount of virus as any other infected cell. On this basis the curve of 7-hour production of virus should take the form of a straight line at an angle of 45° which reaches a maximum level at a value corresponding to one infective particle per cell and running horizontally for larger inocula. The experimental curves obtained are, in fact, only moderate approximations to this form, but they are still close enough to suggest that, by drawing a straight line at an angle of 45° so that it is tangential to the actual curve, we can estimate the dose in terms of HA titre required to give one infecting particle per cell in our preparations. The value is approximately 2 ADs in a volume of 5 ml. For simplicity, therefore, we will assume that when an inoculum at the 2 AD level is used 1 AD of virus is absorbed and effectively infects all the available cells. Deviations from the expected results are a slight increase in yield when the inoculum is larger than the amount capable of infecting all cells and a failure of small inocula to give the yield to be expected. The first would be expected to result from the fact that infection is far from simultaneous so that an excess of virus would ensure all cells being infected considerably earlier than would be the case if only the minimal number of virus particles needed were present. The tailing off with small inocula can be ascribed to the relatively greater effects of adsorption and inhibition processes on smaller amounts of liberated virus.

We shall adopt the view that with our techniques infective fluids behave *as if* they contained in each 5 ml. n particles per HA unit where n is the number of susceptible cells in the DEE preparation. We assume further that, depending on their functional state, the same units are concerned in all the biological effects on the cell that we shall postulate.

On this basis we can at once exclude multiplicity reactivation as an adequate explanation of the undue production of HA. Once the degree of incompleteness exceeded the 1:100 level no haemagglutinin should be produced with an inoculum incapable of averaging about two particles a cell, i.e., less than 4 AD

per DEE, and with increasing degrees of incompleteness, where a multiplicity of more than two would presumably be needed, the curve should swing even more to the vertical. There is nothing in the experimental results to exclude the possibility that multiplicity reactivation plays a significant part, but the shape of the family of curves in Figs. 1 and 2 must mean that other processes are more important. We see no escape from the conclusion that haemagglutinin is produced from cells that have been entered by single virus particles which are incapable of initiating infection in the conventional sense.

Such a conclusion has some far-reaching implications. It is convenient to follow Horsfall (1954) as the most recent writer on the subject of incompleteness in influenza virus, in sorting out those implications. We may assume with him that when a cell is entered by a single active particle the new virus liberated from that cell will be all in the form of fully active virus. As soon as it is liberated a process of thermal degradation begins, resulting in a fall in infectivity with little change in the HA titre. As a result, standard fluids harvested at 44–48 hours have already fallen to a fourth of their potential infectivity.

When a dose of fully active virus sufficient to infect all cells with 2 or more particles is inoculated, the virus produced has a low ID_{50}/HA ratio at the time of its liberation. The same holds, according to Horsfall, when one thermally degraded particle and one active particle infect each cell. Both Horsfall and von Magnus speak as if virus particles are either infective or non-infective, but there seems to be no experimental way of differentiating between a mixture of, say, 1 active particle and 99 thermally-degraded inactive particles and a preparation of uniform particles all of which had been so damaged that each had a chance of only 1 : 100 of initiating infection. In view of the fact that there are many situations known in which the titre of a standard virus preparation differs widely according to the host organism used for its titration, we shall accept the second alternative as equally valid. This allows us to add to Horsfall's list the third possibility that degraded virus particles alone may infect cells and give rise to progeny with a still lower ID_{50}/HA titre.

Any attempt to provide a more fundamental interpretation of the processes responsible for the successive stages of incompleteness that the experimental facts seem to require is probably premature. It is possible to elaborate hypotheses in at least two directions. One of our attempts was based on the widely current concept that RNA provides a template for protein synthesis and that in some way the production of incomplete virus indicates a failure of coordination between synthesis of RNA and of virus protein.

A second approach was to elaborate the position wholly from the genetic level and regard degree of completeness as depending on the average number of certain specific genetic determinants in the virus particles of the population in question. By the appropriate postulation of *ad hoc* entities and properties, either of these hypotheses can be made to cover most of the facts, and any eventual

interpretation must obviously make use both of biochemical and genetic concepts. At the present time, however, there is so little known of the nature of protein synthesis that there would be nothing to be gained by elaborating such hypotheses.

The other feature of the experiments that requires comment is the difference between incomplete virus and heat-inactivated virus in the type of interference produced. In our experiments with WS— at a high level of incompleteness, cells infected from the larger inocula must have received a mixture of virus particles including many with no power to produce descendant virus. The presence of these, however, appears to have no effect on the production of haemagglutinin either in the first 7 hours or subsequently. It is only when fully incomplete virus has an opportunaty to be present in the cell alone for some hours that it can produce an interference that will reduce or annul the yield from a second cycle infection. This gives rise to the characteristic dip in the yield at the level corresponding to one nominal particle per cell.

The parallel experiments with virus partially inactivated by heating at 56° C. present an interesting contrast. In most respects the results obtained are in accord with those of many other workers who have studied the interfering activity of partially or completely inactivated influenza virus, and only a brief discussion of the results will be attempted. Haemagglutinin production at 7 hours shows a very steep curve. This suggests that multiplicity reactivation is the important process involved in providing from the larger inocula a yield beyond that corresponding to their conventional infectivity. The experiments, therefore, support the similar contention of Henle and Liu (1951) derived from experiments with virus partially inactivated by ultraviolet light.

The main point of interest, however, is the different shape of the 22-hour curves of haemagglutinin yield. Double infection with active and incomplete virus of various grades appears to allow virtually full production of haemagglutinin over the whole 22 hours. Multiple infection with predominantly heat-inactivated virus, however, interferes seriously with the production of HA between 7 and 22 hours. The experiments are insufficient to allow analysis of the processes which might be concerned, and for the present only the contrast with incomplete virus is recorded.

The persistence of the capacity of incomplete virus to provide genetic components for recombination reactions at a level very little below that of complete virus is in accord with the previous finding (Burnet and Lind, 1954b) that heat-inactivated virus could function in this fashion. Incomplete virus is far from inert when it enters a cell in association with active virus.

SUMMARY.

With repeated passage of influenza virus WS— in deembryonated eggs using large inocula, highly incomplete virus develops and passage fails after 4–7 transfers.

Experiments to determine the first cycle haemagglutinin yield from a graded series of inocula have been made with virus of different degrees of incompleteness. The results show that many virus preparations have a much greater power to produce first cycle haemagglutinin than to give conventional evidence of infectivity.

Evidence that this is not due to multiplicity reactivation is provided by comparison with heated material in which multiplicity reactivation probably does occur.

Incomplete virus can contribute genetic characters to recombinants produced from double infection with an appropriate active virus.

NOTE.—Owing to the departure of Dr. Stevens before much of the work reported was carried out, the other two authors (Burnet and Lind) are wholly responsible for the presentation of the results.

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MORPHINE ANTAGONISM

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In two previous papers (Shaw and Bentley, 1949, 1952) we have described the powerful analeptic activity of a number of compounds. These substances have the property of arousing dogs from a deep narcosis caused by morphine, or by morphine plus hyoscine. The arousal is very rapid and restores the animals to a condition of complete awareness and of good muscular coordination. This paper describes the action of these analeptic compounds.

Only two other compounds are known which antagonize morphine, i.e., N-allylnormorphine and N-allylnorcodeine (Unna, 1943). Both these compounds are structurally related to morphine. However, the compounds we have described are not in any way related to morphine, nor are all the members of the series related to each other.

METHODS.

Healthy dogs of both sexes were used. Morphine 10 mg./kg. with or without hyoscine (0·6 mg./kg.) was given by subcutaneous injection, usually into the loose skin of the flank. In most cases, this produced a very deep narcosis during which minor surgery could be performed. This narcosis would normally last for at least 8 hours. Resistance of varying degree was occasionally encountered, especially when morphine alone was used. But unless a deep sleep was obtained, the animals were not used, as it was observed that further doses of the narcotic seldom increased the depth of sleep.

The compounds to be tested for analeptic action were administered in most cases intravenously (I.V.). A few drugs were also tested for activity after intramuscular injection (I.M.). They were always given as salts, but the dosage was calculated on the content of free base.

The animals were observed for signs of arousal for at least 1 hour, and if these appeared, they were watched for a further period of 4 hours or more in case there was a relapse to unconsciousness. Since the narcosis produced by the morphine or morphine plus hyoscine was not always of a uniform depth, assessment of the analeptic activity must necessarily be somewhat subjective. A "good arousal" was claimed when the dog not only became alert and sat up, but also walked voluntarily. When the dog sat up of its own accord, but walked only when called or gently moved, a "mild arousal" was recorded. A compound was classed as "inactive" when it failed to produce sufficient increase in awareness for the dog to sit up and make some attempt to walk when gently prodded, for 30 minutes after the injection of the drug.

Subsidiary experiments were carried out on cats and rats. It must be remembered that of the common experimental animals, only dogs are narcotized by morphine. The response of cats is "bewilderment," of rats catatonia.

CHEMICAL MATERIAL.

Group (i). The compounds tested, together with certain physical constants, are listed in Table 1. They fall into two groups, i.e., (a) compounds chemically related in some way to 5-aminoacridine, and (b) compounds showing parasympathomimetic activity.

Albert, Rubbo *et al.* (1945) have discussed the inter-relationship between structure, basicity, antibacterial activity and toxicity in a large series of substituted acridines and related compounds. They have shown that both mammalian toxicity and antibacterial activity are aligned with a high basicity, associated with the "extra-ionic resonance" between the ring nitrogen and the amino substituent. We were not able to investigate all the members of so large a series. But since we have tested members from both the highly ionised and the poorly ionised groups, we feel that some generalisations are possible. The first drugs examined were all monoaminoacridines, i.e., 5-aminoacridine, or monacrin, tetrahydro-5-aminoacridine (T.H.M.) and 1-methyl-5-aminoacridine. These are all highly ionised compounds. Of the other monoaminoacridines, only the 2-amino compound was available, and this was in short supply. It also has a high pKa. None of the 3 poorly ionised monoaminoacridines was available. Two diaminoacridines were tested, i.e., rivanol and proflavine. They also have a high basicity. Amino-quinolines and -pyridines were next investigated. Of the quinolines, only the 4- and 5-amino derivatives were available. 4-aminoquinoline is a strongly basic compound (pKa=9·1), while 5-aminoquinoline has a pKa of 5·4. 4-aminopyridine is a strong base, and 2-aminopyridine has a pKa considerably lower.

Since analeptic activity was found in aminopyridines, analogous aminothiazoles were also tested. These were 2-amino- and 2,4-diaminothiazole, and 2,4-diamino-5-phenylthiazole. However, although analogous derivatives of thiazole and pyridine usually show marked chemical similarity, 2-aminothiazole and 2,4-diamino-5-phenylthiazole have pKa values much lower than 2-aminopyridine.

Only one synthetic compound with a nucleus larger than the acridines was tested for analeptic action. This was 2-amino-3:4-benzcinnoline, with a pKa of 6·68 (Gore and Phillips, 1949).

Group (ii). All the active analeptics in the series described above showed marked parasympathomimetic activity. This has been shown to be associated in some cases with a powerful anticholinesterase activity, although some compounds which produce signs of parasympathetic stimulation are without action on cholinesterase (Shaw and Bentley, 1953). Therefore, it seemed important to test other compounds, chemically unrelated to the substances in group (i), but pharmacologically similar in having parasympathomimetic activity. Accordingly, the alkaloids eserine, arecoline, pilocarpine and nicotine were tested, as well as the following heterogeneous group: potassium chloride, diisopropylfluorophosphate, neostigmine and carbaminoylcholine.

RESULTS.

Dog.

The compounds of greatest interest are described below in some detail. The less interesting members are mentioned only briefly in Table 1.

(i) *Aminacrin* (5-aminoacridine, "monacrin"). This compound has not continued to produce the uniformly good results originally reported (Shaw and Bentley, 1949), although it is still recognized as a powerful morphine antagonist. Since the original work, eighteen more dogs have been tested with aminacrin, 5 having morphine alone, and 12 having morphine plus hyoscine. The monacrin was given in doses of 5 mg./kg.

Six dogs showed "good arousal" and 7 showed "mild arousal," frequently delayed beyond the usual 2–4 minutes, 5 were not aroused at all by aminacrin, although two of these were shortly afterwards aroused with eserine.

No satisfactory explanation can be advanced for this failure to live up to the early high promise. Even more remarkable is the alteration in qualitative effects. The earlier cases all showed great excitement when aroused. This was seldom observed in this later series of 18 dogs. The pronounced respiratory stimulation of the earlier series was similarly less marked.

(ii) *1,2,3,4-tetrahydro-5-aminoacridine (T.H.M.).* T.H.M. appears to be the most active analeptic tested. It was previously reported that it produces a high degree of mental clarity in dogs which have been aroused by its action. With most other analeptics there is usually a period of about one hour's duration when the aroused animal shows some mental confusion and fear of humans. This is seldom seen when T.H.M. is used. The aroused dog, except for some restlessness and muscular weakness, appears normal, usually comes when called, and shows other signs of friendliness.

Twelve more dogs have been tested. Two were narcotized with morphine alone and ten with morphine plus hyoscine. The analeptic was given in doses of 5 mg/kg. I.V. One dog showed a mild arousal only, and ten others showed a dramatic return to activity. The only failure occurred when the T.H.M. was given subcutaneously.

Occasionally mild convulsions have been observed after T.H.M. These occur within one minute of the injection and last at the most for 2 or 3 minutes. After that the dogs appear normal. The very pronounced respiratory stimulatory action of T.H.M. has been confirmed.

(iii) *Rivanol.* Five dogs, all narcotized with morphine alone, have been tested with this compound, which was given in doses of from 10 to 40 mg./kg. At 40 mg./kg. 2 dogs showed good arousal. The effect of rivanol differs from most of the other compounds in that it produces no excitement at all.

(iv) *4-Aminoquinoline.* Three dogs were tested with this compound, all were narcotized with morphine alone. One dog showed "mild arousal" after 5 mg./kg. I.V., but relapsed to drowsiness after a short time. A further 5 mg./kg. of the analeptic produced a "good arousal" with some restlessness. The other 2 dogs were rapidly and completely aroused with a single dose of 5 mg./kg. They showed marked restlessness.

(v) *4-Aminopyridine.* This compound in doses of 3–5 mg./kg. produced powerful and prolonged convulsions with generalized fasciculations in two dogs which had been narcotized with morphine and hyoscine. At 5 mg./kg. the convulsions commenced immediately, and terminated fatally after 20 minutes. At 3 mg./kg. there was "good arousal," but convulsions commenced suddenly after 15 minutes. As they could not be controlled with chloralose or tridione, the dog was killed.

The third received 2 divided doses of 1·5 mg./kg., five minutes apart. This gave a "mild arousal" at first, progressing through increasing excitement to convulsions after 1 hour. These convulsions were controlled with pentobarbitone, but the dog died 2 days later after an apparent recovery.

The other 2 dogs received 2 and 1·5 mg./kg., respectively. Both showed "good arousal" after 5–10 minutes, preceded by marked muscular fasciculation, reminiscent of the effect of large doses of eserine.

Once again a marked but transient stimulation of the respiration was noted. However, this compound, while showing good analeptic action, is too convulsant to be recommended for any clinical application.

(vi) *2,4-Diamino-5-phenylthiazole.* 2-Aminothiazole and 2,4-diaminothiazole both have slight analeptic action. However, the addition of a phenyl group to the latter compound produces a very marked increase in activity.

Fifteen dogs were given morphine plus hyoscine, followed after about 1 hour by 15–20 mg./kg. of 2,4-diamino-5-phenylthiazole I.V. Three showed "mild arousal" only and the remaining twelve showed "good arousal." No failures were noted in this series.

This compound does not produce as marked stimulation of the respiration as do aminacrin, T.H.M., etc., but is still a powerful respiratory stimulant. It is a very reliable analeptic and possesses the advantage that it has not produced convulsions.

(vii) *Eserine.* This drug was the first compound in the series which had previously been used in medicine.

Twenty-three dogs have been used in testing eserine. Six dogs were narcotized with morphine alone, the others with morphine plus hyoscine. The analeptic was given over a wide dose range, i.e., from 3 mg./kg. I.V. to 0·25 mg./kg. I.M. (eserine, together with T.H.M. and 2,4-diamino-5-phenylthiazole, is active when given by the intramuscular route).

Twelve dogs showed "good arousal," five showed "mild arousal" and the remaining six were classed as failures. Sixteen of the above series were given the eserine by the intramuscular route (0·25–1·0 mg./kg.), and of these twelve were classed as successes.

The effects of eserine differ qualitatively from the other more active analeptics in producing no signs whatsoever of excitement, and the respiratory stimulation is very slight. It is interesting to note that Slaughter and Gross (1940) have shown that eserine and neostigmine can potentiate the analgesic effects of morphine.

(viii) *Neostigmine.* As eserine had shown high activity it seemed important to test neostigmine. It was a great surprise to find that in the three dogs tested this drug was quite inactive in doses of 0·5 mg./kg. I.V.

(ix) *Nicotine.* The action of nicotine is especially interesting. It is the only analeptic which will produce arousal followed frequently by a relapse to

unconsciousness some time later. After doses of 1–2 mg./kg. I.V., arousal was noted in 9 of a series of 10 dogs. It commenced 8 to 15 minutes after the

TABLE 1.

Compounds tested for analeptic activity.

Compound	pKa	Dose mg./kg.	Effect	Comments
Group 1.				
(a) *Acridines*				
5-aminoacridine ("monacrin")	9·9	5	good arousal	moderate excitement and resp. stim.
1-methyl-5-aminoacridine	10·2	5	good arousal	moderate excitement and resp. stim.
1, 2, 3, 4-tetrahydro-5-aminoacridine (T.H.M.)	9·9	5	v. good arousal	pronounced excitement, good mental clarity, strong resp. stim.
2-aminoacridine	8·2	3	good arousal	little excitement
5-ethylaminoacridine	9·5	2·5-6·5	good arousal	produced strong convulsions then rapid arousal, with rage and prolonged restlessness
5-morpholineacridine	8·2	2-5	no arousal	produced powerful convulsions but no arousal at all
proflavine	10·0	5-20	no arousal	—
rivanol	11·0	10-40	good arousal	no excitement
(b) *Cinnoline*				
2-aminobenzcinnoline	6·7	5-8	no arousal	—
(c) *Quinoline*				
4-aminoquinoline	9·1	5	good arousal	produces restlessness and resp. stim.
5-aminoquinoline	5·4	5	no arousal	—
(d) *Pyridines*				
2-aminopyridine	6·8	5	good arousal	some resp. stim. brief convulsions
4-aminopyridine	9·1	1·5-5	good arousal	powerful convulsions, prolonged at higher dose, good resp. stim.
(e) *Thiazoles*				
2-aminothiazole	5·4	20-40	mild arousal	mild resp. stim.
2, 4-diamino-5-phenylthiazole	7·0	15-30	v. good arousal	mild resp. stim., little excitement
Group 2				
(a) *Alkaloids*				
eserine		0·25-3·0	v. good arousal	little resp. stim.
nicotine		1-2	good arousal then relapse	relapsed animals could be roused again with T.H.M., etc.
arecoline		0·25	no arousal	—
atropine		20	deepens narcosis	—
pilocarpine		5	no arousal	—
(b) *Other parasympathomimetic drugs*				
carbaminoyl choline		0·1-0·5	deepens narcosis	
diisopropylfluorophosphate		0·2	no arousal	
potassium chloride		15-22	no arousal	
neostigmine		0·5	no arousal	

injection of nicotine, and the relapse occurred 20–30 minutes later. Four of the "relapses" were subsequently aroused with T.H.M.

Other compounds tested are listed in Table 1.

In summarizing this section we may say that T.H.M. is the most active analeptic in the series. It produces a high degree of mental clarity and bodily activity. Eserine and 2,4-diamino-5-phenylthiazole are slightly less dramatic but appear to be very reliable. All three compounds are active when given by the intramuscular route.

Cat.

Control intraperitoneal and intravenous injections of morphine in the cat. It was found that injections of morphine of up to 20 mg./kg. invariably caused excitement after a period of about fifteen minutes. The condition was accompanied by dilated pupils and lasted for some hours. In no case in which morphine was given alone were any convulsions noted (ten cats).

Control injections (intravenous and intraperitoneal) of aminacrin. Three cats were given aminacrin intravenously (10 mg./kg.). This caused immediate weakness and in a minute a brief but sharp convulsive seizure. The animal recovered from this in about one minute, and during the next twenty minutes it seemed panicky and angry, but otherwise normal. Larger doses (15 mg./kg.) produced severe convulsions.

Aminacrin in morphinized cats. Intraperitoneal injections of morphine (10 to 20 mg./kg.) provide in about twenty minutes sufficient analgesia to permit exposure of the saphenous vein. In four of the five cats intravenous injections of aminacrin (10 mg./kg.) caused an increase in wildness, which progressed to violent convulsions. Death supervened up to one hour later. The convulsions were accompanied by profuse salivation and widely dilated pupils.

Morphine in cats under ether anaesthesia. The cats were anaesthetized with an ether-chloroform mixture, and a cannula was inserted in the saphenous vein. The morphine was given *via* the cannula, just as the cats were regaining consciousness. Invariably this produced a deep sleep without any signs of excitement at all. It is a remarkable fact that whereas morphine alone excites a normal cat, in one just recovering from ether it produces a further and prolonged state of depression. About twenty minutes later the aminacrin was injected (I.V.). In all, fourteen animals were treated in this way, and of these seven showed a rapid and pronounced arousal when aminacrin (10–15 mg./kg.) was injected. Of the remainder, two showed some signs of arousal. Of those aroused, four developed convulsions and died from one to three hours after the aminacrin injection.

Thus it may be stated that aminacrin potentiates the excitant action of morphine in the cat, and in 60 p.c. of the cases studied it was found to have a good analeptic action against the narcosis produced by a combination of ether

and chloroform, plus morphine given intravenously. A pronounced increase in respiration was noted every time the aminacrin was injected intravenously.

The highly excitant action of intravenous aminacrin is also seen in the rabbit, where aminacrin can precipitate typical morphine convulsions much earlier than one would expect without the aminacrin, and after doses of morphine that would not normally cause convulsions.

Rat.

The effect of morphine on the rat is to produce catatonia, and a true narcosis seldom develops. To investigate this phenomena a group of four rats was given approximately 30 mg./kg. morphine intraperitoneally. Catatonia appeared in ten to fifteen minutes and an injection of 4 mg./kg. T.H.M. was then given I.P. This produced an abolition of the catatonia in all the rats.

The 2-aminopyridine produced, in normal rats, a marked increase in activity, which appeared as bursts of running. The catatonia following morphine was little effected.

Rivanol had no action on the catatonia.

Nicotine had no effect on the catatonia, in contrast to its marked action in the morphinized dog.

DISCUSSION.

In spite of the fact that we have been unable to obtain and test a number of compounds in Group 1, we can make the following generalizations.

(i) High analeptic activity does not invariably accompany high basicity. The active alkaloids are all weakly basic substances, and so are 2-aminopyridine, 2-aminothiazole and 2,4-diamino-5-phenylthiazole. Furthermore, the highly ionized compound proflavine is inactive.

(ii) Although a number of the more active analeptics show potent anti-cholinesterase activity, this is not a necessary condition for activity. 4-amino-quinoline produces only 8 p.c. inhibition of the enzyme at a concentration of 10^{-4} M., and 2-aminopyridine, 2-aminothiazole and 2,4-diamino-5-phenylthiazole are without action at this concentration.

(iii) All the active analeptics so far tested have fairly strong parasympathomimetic action. This may be either a direct "muscarinic" action, or an indirect effect, *via* the action on cholinesterase.

(iv) All parasympathomimetic drugs are not analeptics. For example, neostigmine, carbaminoyl choline, D.F.P. and potassium chloride are all inactive. Schweitzer and Wright (1937) have attempted to explain the differences in the action of eserine and prostigmine on the central nervous system. They believe that eserine, with its tertiary nitrogen, would easily pass the cell membrane, while the more strongly basic quaternary nitrogen of prostigmine hindered its entry into the cell. This concept cannot be used to explain the differences in

analeptic activity. Many active members in Group 1 are large flat molecules with a high basicity. It is difficult to imagine them readily entering a cell.

(v) The features which Albert *et al.* (1945) have shown to be necessary amongst the acridines for antibacterial action are not necessary for analeptic action. For antibacterial activity in the series, a large flat molecule is required with a high basicity. We have shown analeptic activity in small molecules, i.e., aminopyridine, in "buckled" molecules, i.e., T.H.M., and in poorly ionized molecules, i.e., 2-aminopyridine, 2-aminothiazole and 2,4-diamino-5-phenylthiazole. Furthermore, one active antibacterial has been shown to be devoid of analeptic activity, i.e., proflavine.

Antagonism to morphine does not parallel activity against barbiturates. Monacrin and 4-aminopyridine both appear to potentiate the toxicity of pentobarbitone in cats and rats. It is probably significant that morphine and the analeptics are all cationic compounds, while the barbiturates are anionic.

(vii) While many of the active analeptics show some degree of convulsant action, these two properties do not always run parallel. 5-morpholineacridine is a powerful convulsant, but is without any analeptic action.

(viii) While the most active analeptics always stimulate the respiration markedly, this feature again is not always noted. For example, eserine does not stimulate the respiration.

We may summarize these generalisations thus: Morphine antagonists are found amongst heterocyclic basic compounds which show parasympathomimetic activity. However, not all compounds of this description are active. We have no means of predicting with certainty which compounds will be active; but it seems probable that other active derivatives will be found amongst analogues and derivatives of 2- and 5-aminoacridines.

SUMMARY.

Certain aspects of the pharmacology of the antagonism between morphine and a series of compounds is discussed.

The antagonism has been investigated in the dog, cat, rabbit and rat, each species behaving in a different manner towards morphine, yet showing similarities in the response to the analeptics.

Most, but not all, of these analeptics have anti-cholinesterase activity. The majority stimulate respiration and produce convulsions. The latter is not, however the means of arousal from morphine narcosis.

The desiderata of an ideal analeptic together with its mode of action are discussed.

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ADDENDUM.

Since writing this paper we have shown that 2,4-diamino-5-phenylthiazole antagonizes pethidine, physeptone and omnipon in the dog. We have also been able (in collaboration with Dr. A. Shulman) to reverse morphine narcosis in humans with tetrahydro-5-aminoacridine and 2,4-diamino-5-phenylthiazole. Full details will be published in a clinical journal.

THE NATURE OF THE SODIUM AND POTASSIUM BALANCE IN NERVE AND MUSCLE CELLS

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Discrepancies in the membrane theory of K accumulation in muscle and nerve cells led us to re-examine the behaviour of these tissues when soaked in Ringer's solution under various conditions. This work gained further impetus from the recent paper of Ling (1952), who has postulated a selective ionic adsorption to account for the high K content of these tissues.

The use of radioactive isotopes has disproved any theory involving specific ionic impermeabilities. Subsequent to this, the principles underlying the accumulation of K have centred around the Donnan principle, supplemented by the concept of a Na pump (Dean, 1940; Eccles, 1953).

The main consequences of this hypothesis (apart from electrical phenomena, which will be considered in a later paper) are: (i) the ratio of internal to external K should vary with the external concentration according to a Donnan equilibrium, (ii) K movements into or out of the cell should be balanced by Na movements in the opposite direction, or anion movements in the same direction, (iii) when the cell is dead (and there is no energy to maintain the Na pump) the cell should come to ionic equilibrium with its surrounds, i.e., Na in/Na out =K in/K out and is greater than 1.

That these three criteria are not fulfilled will be evident from the work reported in this paper. This has also been shown by Solomon (1952), who stated on thermodynamic grounds that K movements could not passively result from active Na movements. Lastly, Harris and Maizels (1951) have demonstrated that none of the above criteria apply in the case of the human erythrocyte; i.e., the external concentration of K, within wide limits, has little influence on the concentration of cell K attained after 20 hours; the rate of change of Na content is greater than that of K content, and finally, when the cell is dead, the ratio of external K to internal K only rises to about 0·5 after 4 weeks' standing.

To account for their findings, these authors (and others) postulate the existence of one or more carrier mechanisms for the transport of these ions, and in most cases an attempt is made to link the Na and K shifts.

In this paper we have tested the consequences of the carrier theory either by itself or in conjunction with the Donnan postulates. Our results have led us to discard both these hypotheses and have indicated that a more plausible

explanation would be that the K is held within the cell by a specific adsorption not unlike that of an ion exchange system, but differing from such a non-living resin in certain important features. Ling (1952), Roberts and Roberts (1950), Mazia (1938), Ponder (1949), Harris (1953) have also come to the conclusion that membrane hypotheses are inadequate, and must be replaced by a selective adsorption theory.

METHODS.

In all the experiments to be described we have adhered to the following general principles (unless the contrary is specifically stated). As far as possible the conditions were not varied outside the ranges which should have brought about the death of the tissue, and at the end of each experiment the viability of the tissue was tested; muscles for contractility, nerve for impulse transmission.

It was found absolutely necessary for experiments of a duration longer than six hours to add a mixture of antibiotics to the nutrient medium to ensure sterility. Fungal contamination occurred occasionally, but only results from flasks which were free from fungal and bacterial growth are reported in this paper. The following concentrations were employed:

Penicillin G	10,000 units/litre
Chloramphenicol	10 mg./litre
Streptomycin	10 mg./litre

Control experiments showed that these antibiotics were without effect on the tissues.

Statistics. In all experiments sufficiently large groups of muscles and nerves were taken to permit of statistical analysis. The mean and standard deviation (S.D.) were calculated from the logarithms of the observations as it was found that most of the results were not distributed normally, but could be more accurately treated by a log transformation and subsequent correction of the log mean and log standard deviation as given by Finney (1941) and Sichel (1952). The significance of the difference of means was tested by the "t" test. Where possible, individual pairs were compared, rather than group means. Once again the observations were changed to logarithms. Sometimes an analysis of variance was performed.

Animals. The sartorius muscles and the sciatic nerves of the toad *Bufo marinus* were used in these studies. The animal was available throughout the year in the large numbers necessary for this work, and proved suitable for our experimental procedures. In all, we have analysed 2,000 pairs of nerves and muscles over a period of two and a half years.

Solutions. Since nearly all experiments involved soaking the excised tissue in Ringer solution, the toad plasma was analysed and a modified Ringer constituted, which simulated toad plasma. The mean results of the ionic analyses, carried out on nine samples, each with plasma pooled from three toads, were:

	mM./l.
Na	138·0
K	3·2
Ca	1·9
Mg	0·6
Cl	96·1
HCO_3	23·6
PO_4	0·6
SO_4	4·6

Solutions of the following composition were used throughout this work:

Toad Ringer	mM./l.	Phosphate Ringer	mM./l.
NaCl	115·0	NaCl	115·0
KCl	3·2	KCl	3·2
$NaHCO_3$	20·0	Na_2HPO_4	16·0
NaH_2PO_4	3·0	NaH_2PO_4	4·0
$CaCl_2$	1·3	$CaCl_2$	1·3
Glucose	16·5	Glucose	20·0

Aeration of the Toad Ringer with a mixture of 95 p.c. oxygen and 5 p.c. CO_2 maintained the pH of this solution at 7·25, an average which had been obtained from determinations on the plasma of 20 toads.

Procedure. It was found that the ionic content of nerves and muscles varied considerably from animal to animal. The variation between paired nerves and muscles taken from the same animal was much smaller, so one of each pair was routinely used as a control, while the opposite partner was subjected to the experimental conditions to be outlined.

The nerves or muscles when removed, either from the animal or from the Ringer solution, were blotted, weighed and dried at 105° C. Volume changes were followed by weighing the tissue immediately after removal from the animal and at the end of the soaking period. The dried nerves and muscles were ashed in an electric muffle furnace at 560° C. for 6 hours. The ash was taken up in N/40 HCl and the Na and K content estimated with a Beckman flame spectrophotometer.

EXPERIMENTAL.

Normal content of Na and K. In most experiments from 6 to 8 pairs of nerves and muscles were used. Initially, the variation between the members of each pair was investigated by analysing the tissue immediately after removal from the animal. The degree of variation between pairs, with its S.D., is shown in Table 1.

TABLE 1.

Normal Ionic Content

	Na mEq/Kg.		K mEq/Kg.	
	Dry Weight	Wet Weight	Dry Weight	Wet Weight
Sciatic	281·6±19·7	63·4±3·5	196·0±11·6	37·3±0·5
Difference between pairs p.c. of mean	9·9	9·9	9·2	8·1
Sartorius	142·6±18·4	26·3±3·1	478·2±20·7	91·8±2·8
Difference between pairs p.c. of mean	6·3	7·2	6·1	5·3

Temporal variation. Analyses of groups of nerves and muscles have been carried out at intervals over a period of two and a half years. This has enabled us to detect certain temporal differences between groups. The level of Na and K in sartorii remained comparatively constant over the whole period, but the sciatic nerves showed marked fluctuations in the level of Na from time to time. The K content did not show a similarly great fluctuation. The differences in the Na level amounted in some cases to 100 p.c. and were consequently too great to be regarded as experimental error. Figs. 1 and 2 give an indication of the variation in ionic content of nerves and muscles over a period of two and a half years.

In general, the Na content tends to show a fall in autumn, which is more marked in nerve than in muscle. But it is not generally possible to associate

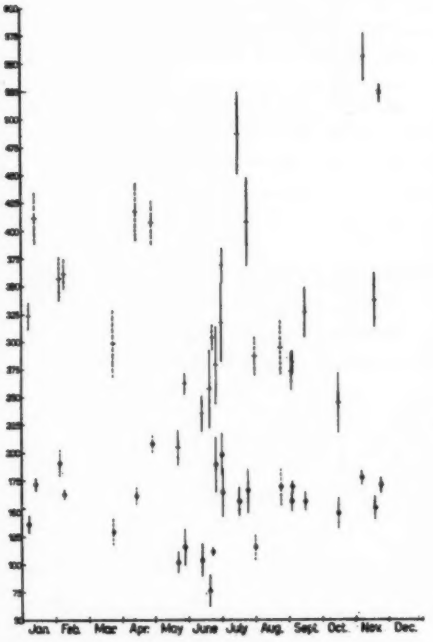

Fig. 1. Temporal variation in sodium and potassium content of sciatic nerves.
Sodium mEq/Kg dry weight: +.
Potassium mEq/Kg dry weight:
Solid lines, 1952; dotted lines, 1953; dashed lines, 1954.
The vertical lines indicate confidence limits at a probability of 5 p.c.

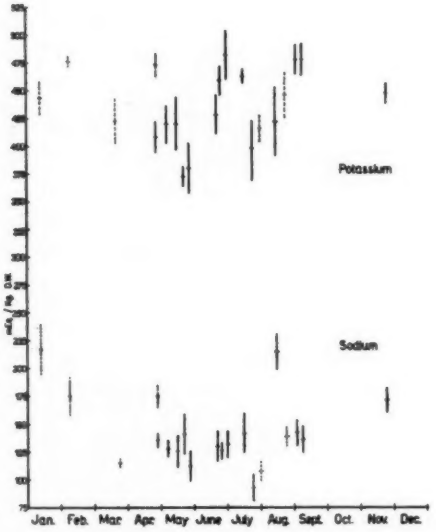

Fig. 2. Temporal variation in sodium and potassium content of sartorious muscles.
Solid lines, 1952; dashed lines, 1954.
The vertical lines indicate confidence limits at a probability of 5 p.c.

all the fluctuations with the seasons, possibly due to the intervention of abnormal climatic conditions (as the toads were collected 1000 miles from our laboratory, we were unaware of their climatic environment).

The effect of hydrogen ion concentration.

As is well known, and as will be shown fully in the next section, excised tissues tend to lose K and gain Na when soaked in normal Ringer solution, but this process is checked after the exchange of a small fraction of the ions. It has been suggested that the separation of ions against an electro-chemical gradient is due to an active transport of either or both Na and K ions. If the active transport is an enzymatic process, then the ionic levels in the cell should vary with the pH of the external medium.

Groups of 6–8 muscles and nerves were soaked in Ringer solution of pH varying from 4·8 to 9·15, for from 4–6 hours for sartorii, and from 10–14 hours for sciatics. The Na and K content of these tissues was compared with controls analysed immediately after removal from the animal, and the loss or gain of these determined. The results which have been obtained may be ascertained from Figs. 3 and 4.

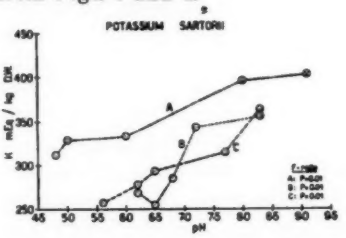

Fig. 3. The effect of pH on potassium content of sartorius muscles.

Each point of the lines A,B,C is the average of at least six muscles, and each series was carried out on the same day. The analysis of variance shows that the regressions are significant.

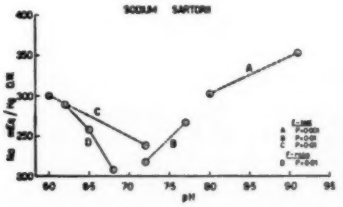

Fig. 4. The effect of pH on sodium content of sartorius muscles.

Each point of the lines A,B,C is the average of at least six muscles, and each series was carried out on the same day. The "t" test or analysis of variance shows that the regressions are significant.

The first striking feature of these curves is the different effect of hydrogen ion concentration on the loss of K and gain of Na by muscles and nerves. We propose to discuss the muscle series first as the results with this tissue showed a greater constancy. The passage of Na into the fibre of soaked muscle was at a minimum at pH 7·25 (the pH of toad plasma) and increased steeply at higher or lower hydrogen ion concentration.

The curve for the loss of K is quite a different shape from that of the Na gain. The loss of K from the muscle fibre decreases with increasing pH. The curve obtained is fairly steep below pH 7·25, and tends to flatten out on the

alkaline side. The fact that there is no reciprocal relationship between the K and Na curves would eliminate two usual explanations of the phenomenon. The first is that the retention of K is the result of a Donnan equilibrium which is itself sustained by an active extrusion of Na (the Na pump). Certainly the appearance of the Na curve, with an optimum extrusion at physiological pH, suggests the presence of an enzyme carrier. The second explanation discards the Donnan equilibrium entirely, and postulates the existence of an enzymatic pump for each cation. But the shape of the K curve permits of no such explanation since it is difficult to conceive of an enzymatic reaction which does not have a point of optimum activity at a particular hydrogen ion concentration. If we assume, however, that the K ions are retained in the cell by binding on to the cell protein or other substrate, as is found in an ion exchange resin, the shape of the K curve may be interpreted as an effect on the ability of the protein to discriminate between Na and K in its cation binding. At high hydrogen ion concentration the protein loses its specificity with regard to K binding, and Na enters passively, along its concentration gradient. At physiological pH and above the protein binds K at its maximum efficiency. Superimposed on this is the general increase in base binding of the cellular proteins at higher pH, which produces the curve of increased total cation with decreasing hydrogen ion concentration.

As mentioned above, variation in the hydrogen ion concentration of the Ringer solution in which nerves were soaked had an effect quite different from that in muscle. Nerves which had a high initial level of Na were capable of extruding this Na into the Ringer against a concentration gradient. Those with a low initial Na content resembled muscle in their behaviour, in that they took up Na. A few pairs of nerves showed no change on soaking. Nerves also differed from muscles in that soaking in Ringer did not cause a loss of K. At physiological pH and higher there was a slight non-significant gain in K against a concentration gradient. At any pH lower than pH 6 a small amount of K was occasionally lost from the nerve. Here again the independent nature of the Na and K movements rules out the possibility of a Donnan type system; and the lack of well-defined pH optima indicates a stable adsorption system, rather than two metabolically powered "pumps."

Despite large variations in external hydrogen ion concentration, no significant changes in tissue volume were noted in this experimental series.

Rate of ionic movements upon immersion.

Soaking the sartorius muscle in Ringer solution causes a considerable loss of K and gain of Na, and it was decided to investigate the rate at which these changes take place. Groups of 6–8 pairs of muscles were taken, and one of each pair soaked in Ringer for a certain time. The muscle was weighed and analysed and the Na and K level compared with that found in its companion

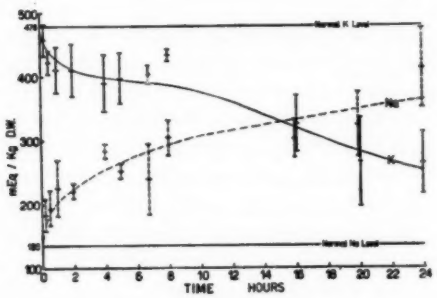

Fig. 5. The effect of time of soaking in normal Ringer on sodium and potassium content. Normal sodium and potassium level refers to ionic content of unsoaked muscle. The vertical lines indicate confidence limits at a probability of 5 p.c.

immediately after removal from the animal. The result can be seen from Fig. 5. Each point is the mean of at least six pairs. It will be seen that after an initial rapid movement of ions a state of equilibrium is reached, which is maintained for about 20 hours. The initial entry of Na precedes K loss, but after half an hour they become approximately equivalent. It must be emphasized, however, that it is only the average gain or loss which is equivalent. In individual cases there may be large discrepancies, which are statistically significant. That is, a large gain of Na is not always accompanied by an equivalent K loss. After twenty hours soaking in Ringer the muscle loses a further amount of K and gains Na. This continues until the tissue fails to contract on stimulation (about 48 hours, at 23° C.). At this stage, although it may be said to be physiologically "dead," the ions have not come to equality with Ringer. A rigorous time curve was not constructed for sciatic nerves, but here again it was found that there was a fairly rapid ionic change, then a period of equilibrium which extended for several days.

Ionic equilibria of "dead" nerve and muscle.

In the previous section it was found that nerves and muscles which failed to respond to stimulation, i.e., were "physiologically dead," were not in ionic balance with the surrounding medium. As the tissue begins to fail to respond to stimulation there is, for the next few hours, a decline in K and an increase in Na. This decline is no doubt due to the fact that a quantum of K is released as each fibre dies; with the death of all fibres a new constant level of ionic content is attained. This equilibrium composition, which contains an elevated K, is maintained for about 24 hours in muscle (Table 2, K 3·2 mEq./l.), and about 3–7 days in nerves (Table 3, K 3·2 mEq./l.). If the tissue were kept for a

period longer than this, the nerve K would fall only slightly, but the muscle K rapidly. Finally, there was an extrusion of muscle K against the Ringer gradient until, in some cases, there appeared to be no K in the fibre. This last interesting stage will be the subject of another communication. However, when nerves were left in Ringer solution for from 6 to 28 hours after they were dead, there was no further loss of K during this period. In one series half were left for 14 hours in Ringer after death, and the corresponding nerve in each pair left for 28 hours. The K level did not alter during the additional 14 hours.

The next step was to investigate the effect of different concentrations of K in the Ringer solution on the level of K retained in the "dead" nerve or muscle. Solutions were made up without K, and with 1/3, 2/3, 2, 4 and 6 times the normal level of K. In each case the findings were compared with the Na and K in the companion tissues of each pair, which was allowed to die in normal Ringer. The results may be seen in Tables 2 and 3. In evaluating these figures it is necessary to consider the K in the extracellular fluid, which will vary as the K level varies in the Ringer. There have been various estimates of the volume of the extracellular space in frog nerve (Shanes, 1952), most estimates

TABLE 2.

Ionic Content of "Dead" Muscles.

All analyses were carried out 18-24 hours after response to stimulation had ceased.

K concentration in Ringer mEq/litre	Na mEq/Kg. Wet weight	K mEq/Kg. Wet weight	K mEq/Kg. in intracellular space
0	112·7±5·1	8·0±2·9	9·8
1·06	82·3±2·5	14·6±4·5	17·0
2·12	79·3±0·5	22·9±3·5	26·6
3·2 (normal)	69·4±9·8	29·1±4·8	33·7
6·4	83·8±1·6	22·1±6·5	24·9
12·8	63·4±4·9	36·2±5·8	40·4
19·2	63·4±8·0	30·5±7·5	32·5

TABLE 3.

Ionic Content of "Dead" Nerves.

All analyses were carried out from 1-3 days after cessation of conduction.

K concentration in Ringer mEq/litre	Na mEq/Kg. Wet weight	K mEq/Kg. Wet weight	K mEq/Kg. in intracellular space
0	90·9±4·0	8·8±1·6	17·6
1·06	120·3±15·3	14·6±3·0	28·2
2·12	92·8±5·8	13·1±0·8	24·1
3·2 (normal)	97·4±9·5	16·1±2·7	29·0
6·4	101·8±5·3	20·7±3·5	35·0
12·8	94·6±10·5	24·3±3·9	35·0
19·2	93·7±6·0	29·1±3·3	39·0

placed it in the region of half the total space. For the sake of convenience we have used this figure. Calculations are further complicated by uncertainty as to the distribution of solids in the intra- and extra-cellular phases of the nerve. The whole nerve contains 16–18 p.c. solid, but it is difficult to decide how much of this is sheath, and consequently extracellular. In view of these difficulties it was decided to abandon the attempt to arrive at an absolute value for the intracellular K, and to compromise by determining the relative differences of "intracellular" K between the groups of nerves. It was assumed, for this purpose, that the intracellular space is equal to the extracellular space, and that the total water is evenly distributed between the two phases. It is emphasized that this will lead to incorrect absolute values, but as the relative size of the compartments, and the distribution of solids, is unlikely to alter under the experimental conditions, the differences should be accurate, and in all probability would not alter the absolute values by more than 10 p.c. Similarly, in the case of muscle, we have arbitrarily assumed that the extracellular space is 15 p.c. of the total, and we have made no correction for the distribution of the 18 p.c. solid matter. As the extracellular space is smaller than in nerve, this assumption should lead to an even smaller deviation from the correct absolute value.

Owing to the demand for sterility in these long-term experiments, it was impossible to follow wet-weight changes in all cases. (It was found that the weighing and transference of tissues from one sterile vessel to another, under laboratory conditions, could not always be carried out without spore contamination.) Where the tissues were weighed on removal from the animal, and after soaking, it was found that the volume changes were very small, random, and could be ascribed to errors inherent in the blotting.

As can be seen from Table 2, increasing K concentration in the Ringer caused an increase in the residual K of the nerve cell. However, soaking the tissue in K-free Ringer did not cause a loss of all the K, nor was the increase in intracellular K proportional to the increase in Ringer K, as would be expected if the K retention were the result of a Donnan equilibrium.

The so-called "dead" muscles retained about a quarter of their total K. The intracellular K was calculated, and was not significantly altered by altering the K level in the Ringer solution, nor did soaking the muscle in K-free Ringer reduce the level to zero.

The effect of metabolic inhibitors on nerve and muscle.

In the previous section muscles and nerves were described as "dead" when they no longer contracted or produced an action current on electrical stimulation, respectively. This may be referred to as physiological death. When, however, either tissue was placed in a Warburg apparatus at the end of this period there was usually some oxygen uptake. Thus the tissue was not biochemically

dead. In order to inhibit completely glycolysis and respiration, the tissues were immersed in Ringer solution containing a mixture of cyanide and iodoacetic acid. This experiment was carried out in two different ways: in the first instance the nerves and muscles were allowed to die in antibiotic Ringer, containing cyanide and iodoacetic acid, one of each pair was then placed in a Warburg apparatus to determine if there were any O_2 uptake, the other was analysed. The results of this experiment are collected in Table 4. It is evident from this Table that muscles which had a low O_2 uptake always contained K in excess of that in Ringer (these muscles were physiologically dead). However, many muscles which were also biochemically dead, and consequently had no O_2 uptake, also contained K at a concentration at least three times that of Ringer. Results, to be reported later, indicated that the K content of "biochemically" dead muscles remained elevated for about 30 minutes before declining. The scatter of the K determinations (as shown by the large standard deviations) is due to this rather rapid decline shortly after death and not to experimental inaccuracies, a contention supported by the evident accurracy of the Na determinations.

A confirmatory procedure was as follows. The muscles and nerves were set up in a Warburg apparatus, and the O_2 uptake followed at 10-minute intervals.

TABLE 4.

Ionic Content of Sartorii poisoned with NaCN (25 *mM*/l) *and IAA* (8 *mM*/l).

Na mEq/Kg.		K mEq/Kg.	
Dry Weight	Wet Weight	Dry Weight	Wet Weight
492	94·2	74·2	14·2
510	100	45·0	8·9
485	91·0	71·6	13·5
472	98·1	65·5	13·6
625	105	38·1	6·4
682	102	71·0	10·6
566	94·0	38·2	6·5
Mean 550±51	98·4±3·1	56·7±19·8	10·3±2·2
612	109	87·5	15·5
600	108	106	19·0
605	86·3	27·5	3·9
726	92·3	32·9	4·2
715	117	112	18·5
638	95·5	44·8	6·7
510	91·0	97·8	17·5
500	83·5	19·3	3·2
485	88·0	168	30·6
Mean 601±54·4	96·6±7·2	70·1±36	11·7±6·9

The first series were without O_2 uptake. The second series showed slight O_2 uptake.

As the tissues died the curve of O_2 usage flattened out. When it was apparent that utilization had ceased (3 hours for muscles immersed in Ringer with cyanide and iodoacetic acid, 6 hours for nerves similarly treated), the tissues were removed and analysed. Once again it was apparent that biochemically dead tissue had not come to ionic balance with the Ringer solution. It was found that if the muscles were not analysed until more than half an hour after biochemical death, then the K began to disappear rapidly from the tissue. The results of these experiments showed that, as the tissues died, the Na approached equality with the Ringer before the K, this would indicate that the extrusion of Na against a concentration gradient is more dependent on the O_2 uptake than is the K retention. Nerves which were poisoned with cyanide and iodoacetate behaved differently from muscles in that they tended to retain K at higher than

TABLE 5.

Na and K Content of Nerves kept in NaCN (25 mM/l) and IAA (8 mM/l). The Nerves showed no O_2 uptake after 6 hours.

Time after death	Na mEq/Kg. Wet Weight	K mEq/Kg. Wet Weight
10 hours	125±3·7	6·8±3·1 } No significant changes
42 hours	129±4·0	7·7±2·1 } between these values.
1 week	126±12·0	11·8±5·3 }

Ringer levels for considerable periods after "death." As can be seen from Table 5, K was still retained in the nerve after a week's immersion in cyanide and iodoacetate.

The effect of cold on the Na and K content of nerve and muscle.

It has already been shown in the section entitled "Rate of ionic movements upon immersion" that muscles, when soaked in Ringer regularly gained Na and lost K, but with nerves the ionic changes were irregular. The effect of low temperature (3°–6° C.) on these movements was now investigated.

Muscles. Groups of 6–8 muscles were held at 3° C. for 4 hours, and the Na and K content was compared with the companion muscle which was kept at 23° C. The results are shown in Table 6, and indicate that the loss of K and gain of Na found at 23° C. is accelerated in the cold. The Na gain tends to be greater than the K loss. These results were compared with those obtained on cooling two groups of muscles for 2 and 4 hours respectively, when it was found that those held at 3° C. for 4 hours had lost significantly greater amounts of K, and gained more Na, than the group cooled for two hours before analysis.

An attempt was made to reverse these ion movements by returning the tissues to room temperature (23° C.). Muscles were cooled to 2–4° C. for 4

TABLE 6.

Change of K and Na content of Sartorii on standing 4 hours at 26° C and 6° C.

(Two experiments, 16 muscles in each)				Na gain	Significance	K loss	Significance
26° C		6° C					
Na mEq/Kg. Dry Wt.	KmEq/Kg. Dry Wt.	Na mEq/ Kg. Dry Wt.	KmEq/Kg. Dry Wt.				
246	290	289	261	43	S.	29	V.S.
261	370	317	343	56	V.S.	27	V.S.

NOTE: In this and subsequent tables N.S. means not significant, S. means significant, i.e. probability between 0·05 and 0·01; V.S. means very significant, i.e. probability greater than 0·01.

hours, then one of each pair was analysed. The matching muscle was held at 23° C. for a further 4 hours before analysis. No significant difference in Na and K content was found between either group of muscles, that is warming the muscles had arrested ion movements, but not reversed them. The same results were obtained when the times of soaking were reduced to 2 hours at 3° C., and 2 hours at 23° C.

Active movement of the ions against their concentration gradients was found when the muscles were warmed in Ringer containing K and phosphate at three times the usual concentration. Two groups of muscles were held at 3° C. for 10 hours, the first group was then analysed, and the second transferred to Ringer solution with the elevated K and phosphate content, at 23° C., for 3 hours. Na was found to move out of the cell, against its concentration gradient, and the K level in the cell increased, also against a concentration gradient. The K gain was far in excess of that which would be passively caused by the change in K concentration in the extracellular fluid. The Na extrusion was somewhat greater than the K gain. These active ion movements were further emphasized by reducing the K content of the Ringer solution in which the muscles were

TABLE 7.

Effect of warmth on Na and K content of cooled muscles.

Time at 3° C	Time at 23° C	Na loss mEq/Kg. Dry Wt.	Significance	K gain mEq/Kg. Dry Wt.	Significance	K in Ringer at 23° C	K in Ringer at 3° C
2 hrs.	2 hrs.	+37	N.S.	2	N.S.	3·2 mEq/l	3·2 mEq/l
5 hrs.	4 hrs.	17	N.S.	1	N.S.	3·2 mEq/l	3·2 mEq/l
10 hrs.	3 hrs.	78	V.S.	66	V.S.	9·6 mEq/l	1·0 mEq/l

soaked in the cold to one-third, and then transferring the tissue to a solution containing three times normal K. These results are shown in Table 7. In some cases where sartorii were cooled to 3° C. for 24 hours, and then placed in Ringer containing 9·6 mEq./gm. of K at room temperature for three hours, it was found that Na moved out of the cell against a concentration gradient, but the K level of the muscle was the same as that of Ringer or lower. It would thus appear that there had been considerable loss of K in the cold, which remained unchecked when the muscles were warmed, although the figures showed that there had been an extrusion of Na amounting to as much as 14 mEq./kg. wet weight (see Table 8). It would appear that the mechanism for accumulating and retaining K in the cell had failed, while the extrusion of Na was still taking place.

TABLE 8.

Anomalous Behaviour of 4 Cooled Muscles on Warming in K × 3 Ringer.

Na mEq/Kg. Wet Wt.		K mEq/Kg. Wet Wt.	
5° C	25° C	5° C	25° C
94·3	77·9	20·5	1·0
80·8	71·0	17·9	3·2
95·9	87·4	8·6	2·4
100·6	86·7	13·9	2·5

Nerves.

These experiments were duplicated using the sciatic nerve, and essentially similar results were obtained (Table 9). It was found that nerves which were held in Ringer solution at 5° C. for 12 hours lost K and gained Na when compared with control nerves which had been kept at 25° C. The active movements of the ions on warming the cooled nerves were studied again. The nerves were soaked in Ringer at 2–4° C. for from 8–24 hours, then half were analysed, and the corresponding nerve of each pair held at 25° C. for from 2–16 hours, after

TABLE 9.

Change of Na and K content of sciatics on standing 12 hours at 25° C and 5° C.

25° C		5° C		Change			
Na mEq/Kg. Dry Wt.	K mEq/Kg. Dry Wt.	Na mEq/Kg. Dry Wt.	K mEq/Kg. Dry Wt.	Na gain	Significance	K loss	Significance
415	174	514	138	99	S.	36	V.S.
395	174	442	150	27	S.	24	V.S.

which they were analysed. In most of these experiments returning the tissue to a physiological temperature caused an extrusion of Na and an uptake of K.

In some experiments there was extrusion of Na from the cell which was not accompanied by an uptake of K. Examination of the results, in these cases, showed that the reduction of temperature had not caused a loss of K, which remained at the level found on removal of the nerve from the animal. This difficulty was overcome by reducing the K content of the cold Ringer to one-third normal, and raising the K and phosphate level in the warmed solution to three times normal. The low-K Ringer, in the cold, caused a marked loss of K which was returned to the cell on placing the nerve in the high-K Ringer at room temperature. When these figures were corrected for the additional K in the extracellular fluid, assuming a value of 50 p.c. for this space, it was evident that the K had been returned against a gradient.

At other times the Na was not extruded from the nerve when it was returned to 25° C. The reason for this remains obscure. It would appear that this phenomenon occurs mainly, if not exclusively, in those groups of nerves which had a high initial Na content (see section on temporal variation). These nerves appear either to retain their Na or to take up more when returned to room temperature (Table 10).

TABLE 10.

Effect of warmth on Na and K content of cooled nerves.

Time at 3° C	Time at 23° C	Na loss mEq/Kg. D.W.	Significance	K gain mEq/Kg. D.W.	Significance	K in Ringer	
						at 23° C	at 3° C
18 hrs.	7 hrs.	48	V.S.	13	S.	3·2 mEq/l	3·2 mEq/l
8 hrs.	12 hrs.	133	V.S.	−3	N.S.	3·2 mEq/l	3·2 mEq/l
24 hrs.	7 hrs.	+4	N.S.	11	S.	3·2 mEq/l	3·2 mEq/l
24 hrs.	8 hrs.	26	S.	5	N.S.	3·2 mEq/l	3·2 mEq/l
24 hrs.	12 hrs.	+70	N.S.	25	S.	3·2 mEq/l	3·2 mEq/l
23 hrs.	11 hrs.	+24	N.S.	40	N.S.	3·2 mEq/l	3·2 mEq/l
8 hrs.	20 hrs.	16	S.	24	V.S.	3·2 mEq/l	3·2 mEq/l
8 hrs.	13 hrs.	96	S.	44	V.S.	3·2 mEq/l	3·2 mEq/l
8 hrs.	16 hrs.	+93	S.	22	S.	3·2 mEq/l	3·2 mEq/l
24 hrs.	2·5 hrs.	1	N.S.	36	V.S.	9·6 mEq/l	1·0 mEq/l
10 hrs.	4 hrs.	19	N.S.	41	V.S.	9·6 mEq/l	1·0 mEq/l

DISCUSSION.

The ionic imbalance of nerve and muscle cells is at present held to be the result of a Donnan equilibrium supported by one or more "pumps." In the introduction we have discussed the consequences of this theory, viz.: (1) The ratio of internal to external K should vary with the external concentration; (2) K movements should be balanced by reciprocal Na movements; (3) when the

cell is dead (that is, the pump is no longer functioning), the cell should come to an equilibrium with its surroundings. This equilibrium would be such that the protein and other indiffusible anion would "draw in" Na until the chemical gradient for Na was reversed. The potassium ratio K in/K out should fall until it equalled the ratio Na in/Na out and, according to the amount of fixed anion, would be somewhat greater than one.

The effect on muscle K of varying the K concentration in the external fluid has been studied mainly by Fenn and Cobb (1934) and Boyle and Conway (1941). Much of this work was done at low temperatures, when we have shown that the tissue is not in a state of equilibrium, but steadily loses K and gains Na. The results of the experiments of these authors (and others) are therefore difficult to interpret. A further difficulty is that this work assumed that Na could not penetrate into the cell. When this was disproved the theory was modified by the introduction of a Na pump. This pump, however, would require metabolic energy, which could not be supplied by a cell at 2–4° C. Therefore, we feel justified in neglecting all results which have been obtained with cells in which the Na pump could not be active, and in which the ionic content of the cells had departed considerably from normal (i.e., all those results obtained at 3–4° C.).

When now the few results remaining (i.e., those at room temperature) are considered, they may be summarized by saying that if the concentration of external K is raised more K appears to enter the fibre. This could be due either to a Donnan movement or an adsorption. When, however, one considers that the uptake by the cooled cell is similar to that by the metabolizing cell, it would appear more likely that an adsorption process is operative.

Conversely, when one considers removal of K from the external solution, it has been found that immersing the muscle in a large volume of K-free Ringer does not reduce the intracellular K to zero, as would be expected if it were a Donnan equilibrium. Fenn and Cobb (1934) have shown a small loss of K (3–30 p.c.) when the muscles were soaked in K-free Ringer for five hours at 22° C. Simon (in press), under similar conditions with toad muscle, was unable to show any significant loss compared with controls in normal Ringer.

The uptake of K by a cell in high-K Ringer may be due either to a Donnan movement or adsorption; however, no such choice is left when one considers the behaviour of the tissue in K-free solution. The presence of an almost normal amount of K in such cells can only mean the exclusion of the Donnan hypothesis in favour of one of adsorption. One must always remember that in the current theory the Na pump is an uncontrollable variable. It would be conceivable that, in K-free Ringer, there would be a retention of K if there were an extrusion of Na. No such alteration in Na level has been observed.

It is a further defect of the Donnan theory of K retention that it cannot be applied to vertebrate nerve. It has been shown by Shanes and Berman (1953)

and Feng *et al.* (1950), that increase in extracellular K, even up to isotonic proportions, can produce little or no increase in intracellular K of frog nerve. Similarly, it has been shown (Simon, in press) that soaking toad nerves in K-free Ringer for 17 hours at 23° C. brings about small K losses which can be almost entirely attributed to loss of K from the extracellular fluid. There was no gain or loss of Na from these nerves. These results are in agreement with those of Fenn *et al.* (1934).

In a Donnan system there must be a balance of osmotic pressure on each side of the membrane. Consequently, if one species of ion moves, there must be a reciprocal movement of another ionic species in the opposite direction. Throughout this work no volume changes have been noticed. It would appear that the cell changes its volume only when the ionic concentration of the external medium is changed an unphysiological amount, i.e., changes in external K of more than six times normal. In our experiments analyses for Na and K only have been carried out. Any movement of hydrogen ions or the formation of anions by the breakdown of complex molecules would be unnoticed. We have had many occasions to observe whether this reciprocity of Na and K movements is the general rule. Furthermore, the use of a flame photometer has enabled us to follow K and Na changes in individual muscles rather than to rely on mean changes for each element in separate groups.

In the case of muscle stoichiometrical reciprocity was often observed, but the exceptions outlined below are sufficient to show that this type of exchange is not generally applicable. Even in those experiments in which the group averages showed a reciprocal exchange of ions there were individual muscles where the exchanges were shown statistically not to be stoichiometrically related. Examples of nonreciprocity are instanced by the following: (i) A study of the effect of pH on K and Na movements (Fig. 3) indicates that below pH 7·2 the exchanges are reciprocal, but that above this pH the gain in Na exceeds the loss in K. This may not be an example of true lack of equivalence, but a result of the increased cation binding of the cell protein at an alkaline pH; (ii) a consideration of the changes in concentration of Na and K which take place on soaking the muscles for various times indicates that the initial entry of Na precedes the K loss, but after half an hour the changes become equivalent (see Fig. 5); (iii) a similar consideration of the changes which take place in the Na and K concentration when a muscle is soaked at 3° C. for 4 hours showed that the gain of Na is greater than the loss of K (see Table 6). Conversely, when the cooled muscles are warmed to 23° C. again there is a greater expulsion of Na than uptake of K (see Table 7).

We have just observed that there are examples of nonreciprocity in movements of Na and K in muscle. When we come to consider nerves the examples are even more marked. (i) A study of the effect of pH on Na and K movements indicates that nerves can be divided into two groups, those with

low Na and those with high initial Na content. The behaviour of these nerves depends upon the initial level. The low-Na nerves, when soaked in Ringer over a wide range of pH, gained Na and the K remained constant, the former to the extent of 100 mEq./kg. dry weight. The high-Na nerves also showed no change in K, but there was a loss of Na. This extrusion of Na against a concentration gradient which was noticed frequently in nerve, but never in muscle, would of course require energy. This unexpected result will be the subject of further investigation.

(ii) A consideration of the changes which take place when the nerve is soaked for 12 hours at 3–4° C. showed that the gain of Na is greater than the loss of K. When the cooled nerves were warmed to 23° C. the movements of Na and K were very variable (see Table 10). In only one case in eleven were the Na and K alterations stoichiometrically equivalent, and it was again found (three experiments) that some nerves gained both Na and K.

The final refutation of the Donnan theory follows from a study of ion movements in dead tissue. For under these conditions, which must mean the absence of the Na pump (and all other pumps), the K of the cell should fall nearly to Ringer proportions. That this is not so for nerve and muscle is evident from Tables 2 and 3. It is extremely interesting to note that muscles without oxygen uptake (biochemically dead) can still retain K at a level three times that of Ringer. When the muscle was "physiologically dead" (i.e., contractility absent but with small residual oxygen uptake), the K was retained at a level seven times that of Ringer, and amounting to a quarter of the total initial K of the cell. It is emphasized that the amounts of residual K were constant for a population of muscles, and characteristic of the experimental conditions laid out in the section "Ionic equilibria in dead muscle and nerve." Soon after "biochemical death" the K level in muscle declined to a level lower than that of Ringer, and in some cases fell to zero. The Na level in the cell does not approach Ringer proportions but remains at about 75 p.c. of this level. It could be argued that a residual portion of the Na pump could still be working in the absence of oxidative metabolism, perhaps by an anaerobic source of energy. Such is not the case in nerve where intracellular Na does rise to Ringer level (in poisoned, but not in physiologically dead nerves), although the K level remains at several times Ringer proportions. The muscle K, as we have said, after remaining constant for some hours in a dead muscle, rapidly falls to Ringer level and is then extruded until none is left in the fibre. As the intracellular Na has not reached Ringer level there must be a cation deficit. The replacing cation will be the subject of further investigation. When nerves were soaked in Na depleted solution (Simon, in press) more than one-third of the Na would leave the cell, but the K content remained unchanged. It was only when about 30–40 mEq./kg. (i.e., about half the total Na) of Na had come out that the internal K showed a decrease of about 5 mEq./kg. As the nerve had lost both

Na and K, the movements were in no sense reciprocal. These findings are in line with Shanes (1952), who found similar K losses with Na-deficient solutions. Thus we consider that a Na deficit in dead muscles is not the result of an active Na extrusion, but rather the replacement of a little Na and all, or nearly all, of the residual K by another cation.

The evidence provided by nerves dying either in normal Ringer or after treatment with IAA and NaCN refutes even more strongly the Donnan conception. The K never approaches Ringer proportions even after 7 days in poisoned (IAA and CN) solutions, and whilst the Na does achieve this level, it never accumulates in the cell (see Table 3). It would appear to us, assuming in the dead cell an absence of the Na pump, that the fact that the ratio Na in/Na out does not become significantly greater than one whilst the ratio K in/K out remains considerably higher than one, clearly shows that the dead cell is not a simple Donnan system, but that it shows an ability to bind K in preference to Na, albeit this specificity is less marked in a dead cell than in a live one. It may be argued that in all our experiments a remnant of the Na pump is still active. While estimations of the energy required for the Na pump vary, they are all in agreement that a considerable fraction of the cell's metabolism is used up in this process. We have shown the above results with Na and K to be obtainable in the absence of any aerobic respiration.

An extensive study of the effect of alteration of the external K on the residual K level in dead tissue yielded results which also did not support the Donnan hypothesis. When the K content of the Ringer in which muscles were allowed to die (physiologically) was varied from zero to 6 times K (19·2 mEq./litre) the intracellular K remained constant when the Ringer K varied from 2/3 to 6 times K, and likewise, the internal Na was not altered over this range. It is only when the external K is reduced to 1/3 that we find the internal K to be affected, and it falls to 17·0 mEq./kg. (intracellular space); roughly 5 times the level in normal Ringer. The internal Na is not affected until the tissue is placed in K-free Ringer. As our muscles did not alter in volume the osmotic pressure must have remained constant. The total cation balance sheet shows a deficit of about 30 mEq. when the muscle is dead (see Table 2). An explanation of this is left to a later paper.

The failure of alteration of Ringer K to affect the internal K in dead muscle speaks strongly against a Donnan equilibrium, but even more against the existence, under these conditions, of any pump mechanism. No matter how long after death the muscles are kept the internal Na does not reach Ringer proportions, whilst after a few hours the K declines to zero. That is, the mechanism which "regulates" the Na level in dead tissue cannot have a metabolic background. It is not tied to the K movements, and would appear to be of a non-living or physical nature.

The picture in physiologically dead nerve differs in detail, although it is

probably similar in principle. As one increases the external K sixfold the cellular K is increased by only 25 p.c. The Na level is not affected. Reduction of the external K does not reduce the internal K below the level in normal Ringer until the external level is zero. With zero external K the cellular K is 17·6 mEq./kg. and will remain at this level for up to a week after the death of the fibre. The Na does not alter, nor does there appear to be a cation deficit (see Table 3).

As the relation between internal and external K in dead nerve is almost flat, i.e., increasing the external K 19 times only increases the intracellular K 0·4 times, it cannot be said to support the modified Donnan hypothesis, but would seem to be more accurately described in terms of an adsorption theory.

The evidence obtained by other authors with nerve and muscle is neither extensive nor conclusive enough to support the modified Donnan hypothesis, and our results offer a refutation. The theory has been applied to red blood cells by Flynn and Maizels (1949), but their results were unable to uphold the conception. At present it would appear that the above authors invoke a multiple pump hypothesis to account for the ionic imbalance. Harris and Maizels (1952) have shown that the Donnan ratio $K_i/K_e = Cl_e/Cl_i$ does not apply and that the concentration of Cl_i varies with hydrogen ion concentration and is entirely independent of the metabolic state of the cell and of qualitative changes in the cell Na and K. They also show that the distribution ratio of K between the red blood cell and the external medium, even after four weeks at 4° C. does not approach unity, although Na_i/Na_e does tend towards unity after this period. This would indicate that the red blood cells retain a residual amount of K after metabolism has ceased, in line with our own results with muscle and nerve.

Flynn and Maizels (1949) have shown that an excess of external K has little influence on the concentration of cell K. Also Raker *et al.* (1950) found that the rate of exchange of radioactive K at the steady state is independent of the external concentration. From evidence such as this Harris and Maizels (1952) say that: "The foregoing considerations taken as a whole can hardly be reconciled with an electrostatic explanation based on the movements of K being due to potentials set up by the transport of Na, and they suggest that the uptake of K must depend on carriage by chemical groups from the exterior to the interior."

Such carriage by chemical groups is assumed by these authors to be geared to outward Na transport, but that one to one correspondence in ion movement need not apply. Hodgkin and Keynes (1953) have suggested that K may be drawn into the cell by a secretory process coupled to one which extrudes Na.

Before considering carrier theories in detail, one must discuss certain general principles of this newly postulated mechanism. Although this may be a new theory biologically, it must be considered from the point of view of

established principles of physical chemistry and all suggested hypotheses must be consistent with these principles.

If the ion is carried in chemical combination with the carrier, the complex, until it reaches saturation, must obey the mass-action principle, except in so far as the actual point of equilibrium is altered by the feeding in of energy from the metabolism of the cell. It is difficult to see how a two-way traffic over the same carrier is possible in the presence of an energy source pushing the reaction in one direction. Consequently, the return trip must have a separate carrier or be due to diffusion. The latter has been shown by Solomon (1952) with the red blood cell to be very unlikely, since the external concentration of Na and K does not bear a linear relation to the rate of movement across the membrane.

The only methods of union of the ion with the carrier would be those of chemical bonding or adsorption. It would appear that the former was the method of choice since any degree of desorption requires a marked alteration in environment. Sufficient alteration is unlikely to be experienced as an ion enters the internal environment.

As the ionic movements are against gradients, a source of energy will be necessary. Unless the passage of an ion across the membrane in both directions is dependent on metabolic energy, then lowering the metabolic rate should affect the rate of transfer in one direction only. It has been found by Harris and Maizels (1951) and Solomon (1952) that both transfer constants for the passage of Na across the red-cell membrane fall when the pH is lowered. Raker (1950) showed that reduction of pH lowered glucose utilization rates. Also Mitchell (1953), working with radio-phosphate turnover in *M. pyogenes*, found that alteration of the pH from 7·25, or poisoning with heavy metal derivatives, markedly lowered the flux rate, therefore either carriers exist in both directions or not at all.

A corollary of the carrier theory is the exchange diffusion concept of Ussing (1948-49) and recently employed by Mitchell (1953) to explain the phosphate equilibrium in *M. pyogenes*. This theory will not account for an accumulation of material within the cell, but, according to Ussing, would account for the rapid interchange of Na without any energy requirement. Undoubtedly the most telling point against the carrier hypothesis is made by its own protagonists. Solomon (1952) points out that Ponder (1950) finds that the uptake of K by warmed cells, previously cold-stored, is three times as great as the normal flux observed by himself, and by Raker *et al.* (1950). Hence even complete inhibition of outward transport is not sufficient to account for Ponder's observation.

The evidence for the existence of a pump stems from the experiments of Steinbach (1951) and Maizels (1949), who first cooled the cells to permit an entry of Na, which is extruded against a gradient when the cells are warmed. One variable is however changed, this though mentioned is never stressed, the warm solution contains an excess of K. We also have shown that the "pump"

will not work for muscle unless there is excess K present (see Table 7). The extrusion of Na will require energy, but the alteration of the external K can in no way contribute to this need. It would appear rather that some physico-chemical exchange is taking place between the Na and the K, as the result of a mass-action principle. In other words, the demonstration of the pump action in muscle cannot be made in a normal environment, but only under artificial conditions. In a following paper (Simon, 1955) the conception of the "pump" is criticised more fully. The foregoing has shown that the modified Donnan theory cannot account for the ionic imbalance in muscle cells. On present evidence it may be said that the carrier idea is a logical necessity rather than an experimental fact, indeed there are some facts of a fundamental nature against it. In view of the inadequacy of these theories we would prefer to reconsider the problem, neglecting for the time being all theories of K retention which depend on a selectivity located in the membrane, whether this be in the nature of the membrane itself, or of one or more carriers.

It would appear to us that it would be more reasonable to adopt an adsorption hypothesis. The term "adsorption" is used in much the sense employed by Clark (1952), that no implication is made regarding mechanism. The possible modes of adsorption could range from physical adsorption (as on to charcoal) through exchange resin types to actual compound formation (see also Roberts and Roberts, 1949). It is no more unreasonable to assume that ionic differentiation takes place in the cell interior than that it happens within the mural structure.

The suggestion that K is adsorbed by the cell is not new. Two separate mechanisms have been put forward. Firstly, there may be an unspecified "binding." This would appear to be common in plant cells (Mazia, 1938; Mullins, 1942; Scott, 1944), and has been suggested for red blood cells (Ponder, 1949) and for muscle cells (Harris, 1953). Next we have a more detailed mechanism in the form of an ionic exchange of K for the hydrogen ion. This has been shown for yeast (Conway and O'Malley, 1946; Rothstein and Enns, 1946) and for plants (Brooks, 1939; Orskov, 1948). The paper by Rothstein is particularly explicit in stating that, during active metabolism of carbohydrate, there is an exchange of hydrogen ion from the cell for K from the medium. As a result the cells accumulate K to the extent of 60–80 mM/l. Further, this exchange is specific, the K cannot be replaced by Na, i.e., in the absence of K no exchange takes place, the hydrogen ion diffuses out in company with an organic anion. Thus the ion exchanger is able to differentiate against Na. As it would appear that the plant cells and yeast specifically accumulate K (perhaps by an ion exchange mechanism) it is not unreasonable to apply a similar hypothesis to animal cells. Hodgkin and Keynes (1953) have demonstrated that the mobility of K in nerve fibres from Sepia was little, if at all, less than that of K in free solution. Harris (1954) has reached a similar conclusion with

respect to K movements in frog sartorii. Both the above authors have inferred from this that K cannot be appreciably bound in the cell. However, their conclusions are open to one objection. The half time of incorporation of K in the above tissues varies from 2 to 16 hours (Harris, 1953), yet in these experiments the K was applied to the cell only for a matter of 20 minutes (Harris) or 1–3 hours (Hodgkin and Keynes) before the potential was applied. This time would be insufficient for the incorporation of more than a small fraction of the K within the cell in the case of Harris' experiments, and a somewhat greater amount in the earlier work. We feel that the results of these two experiments should be treated with the utmost caution. Not the least of the objections is the fact that a direct current applied for from one to five hours caused a loss of excitability as the experiment proceeded, as was noticed by both authors.

Roberts *et al.* (1949, 1950) have investigated the uptake of labelled K by *E. coli*. Their results lead them to the conclusion that K is adsorbed on to certain sites in the cell, with a high rate of adsorption and a slow rate of desorption.

At the present time it is not possible to determine in detail the nature of the binding. This has been considered from a kinetic approach by Spiegelman and Reiner (1942), and by Danielli (1952), from the point of view of a co-ordinate linkage. The conclusion is reached that it is unlikely that the selective mechanism is based on linkages Na-O and K-O, though it appears likely that linkages Na-S and K-S might be involved. Roberts *et al.* (1950) consider the binding to be with hexose phosphates, and there is considerable evidence to suggest that K plays a rôle in carbohydrate metabolism (for literature see Roberts *et al.*, 1950).

Recently Ling (1952) has considered in great detail the mechanism by which K might be bound in the cell. He has postulated an ion-binding system based on the fixed negative charges of the cell protein which preferentially adsorb K rather than Na. The uptake of K is assumed to be due to the smaller hydration atmosphere of this ion, permitting a smaller "distance of closest approach" with the negative charges of the protein lattice. The protein adsorbs ATP and hexose monophosphate, and this adsorption leads to the uncovering of fixed negative charges, with which further amounts of K can be associated, thus preventing the association of Na, which is usually able to displace K.

He further discards the membrane theory of retention of large molecules (the so-called "impermeant anions") and produces evidence to show that they can, in fact, freely penetrate the cell membrane. He proposes a system whereby the anions are tethered within the cell by special forces of attraction, and, in turn, adsorb K in virtue of the fact that the dielectric constant between K and a fixed anion would be smaller than between Na and the anion.

The chief objection to this theory as it stands is that Na and K movements

should be reciprocal. It is possible to imagine that K loss might not be followed by Na uptake if there were a reduction in the number of adsorption sites, but it is impossible to account for Na intrusion from the cell which is not accompanied by K uptake. An exception could be made if the Na were displaced by another cation (hydrogen or ammonium ion). Na extrusion without K uptake has been observed by ourselves (Table 7) and by Scott and Hayward (1953) (with *Ulva lactuca*). It has also been shown by Solomon (1952) that exhaustion of glucose from the external medium caused an immediate entry of Na into the red blood cell, while K loss was delayed by several hours. This fact, coupled with our findings and those of Scott and Hayward, would suggest that the energy sources for Na and K movements are different.

In the foregoing we have surveyed the current ideas of K retention and have presented the evidence of other workers to support an adsorption hypothesis. We now wish to consider how our own findings can be fitted to such an hypothesis. In the simplest terms it would appear that cations are bound to anions which are fixed to a cellular structure of undetermined nature. Energy would be required to maintain the specificity of the cellular structure, and in the event of damage or death of the cell this structure is dissolved and the ions are released. In the intact cell we envisage an absolute specificity at the adsorption sites for some at least of the K, and not a competition between this ion and Na. In the past the concept of bound (adsorbed) K has been linked to the view that such K would be osmotically inactive, and also that other colligative properties would be depressed. The point to be stressed is that it is the anion which is fixed in space, and the cation is held by an electrostatic, though specific, bond to the anion. The cation is, however, free to exchange with other cations in its neighbourhood, and, of course, free to move from one anionic point to another under the influence of an electric field. The exact amount of depression, if any, of the chemical activity of the adsorbed K is a matter which will be clarified by an extension of our knowledge of the physical chemistry of adsorbed ions.

SUMMARY.

The normal Na and K content of the sartorius muscle and the sciatic nerve of the toad *Bufo marinus* is given.

Seasonal variations, which are marked in the case of the Na content of the sciatic nerve, are noted.

The effect of varying the hydrogen ion concentration of the external medium on the Na and K content of the above tissues has been investigated.

When muscles and nerves are removed from the body and immersed in Ringer solution there is a loss of K and a gain of Na. The temporal aspects of such changes have been studied.

Tissues allowed to die naturally, or poisoned with iodoacetate and cyanide, do not come to ionic equality with the Ringer solution.

Nerves and muscles in which aerobic metabolism has ceased contain an excess of K and a deficiency of Na compared with the Ringer in which they are bathed.

Muscles and nerves cooled to 3° C. loose K and gain Na more rapidly than tissues held at 25° C. The extent of the changes are also greater.

When cooled muscles are re-warmed to 25° C. there is an uptake of K and an extrusion of Na, but these movements can only take place if the environment is K enriched.

The above findings have been used to refute the current view that K accumulation in the cell is the result of a Donnan equilibrium assisted by a Na pump. When the cell is dead it is assumed that the Na pump is not active. In this situation a cellular Donnan system would require the ratio Na in/Na out =K in/K out to be greater than unity. Actually Na in/Na out varies from 0·7 to 1·0 and K in/K out varies from 3 to 7. Such ratios can be explained most readily by assuming a specific intracellular binding of K.

Criticisms of the conceptions of pumps and carriers have been made in the light of the above results.

An alternative hypothesis to the contemporary theory of K accumulation is proposed. The accumulation is the result of a specific electrostatic binding to anions fixed to a cellular structure of undetermined nature. A source of energy is required to maintain the specificity of the cellular structure, and, in the event of the death of the cell, this structure is disorganized and the ions are released. The nature of the binding is such that the ions may be osmotically active.

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THE EFFECT OF YOHIMBINE ON SODIUM AND POTASSIUM MOVEMENTS IN RESTING NERVE AND MUSCLE

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In a previous publication MacCallum *et. al* (1949) reported the readily reversible inhibition of conduction in nerve and muscle brought about by yohimbine. When these tissues are treated with this drug and stimulated repetitively with a frequency of about 10 per second, it is found that the first few impulses will be transmitted but subsequent impulses are blocked. If the stimulation frequency is reduced to one per second this block does not occur. It would appear that the recovery processes which take place after the passage of an impulse have been slowed. So one may take advantage of this retardation by yohimbine to investigate the changes taking place during conduction.

The rôle of ion movements in the maintenance of the resting potential and the propagation of the impulse is an integral part of modern electrophysiology (Eccles, 1953). Briefly, the resting potential is held to depend on a high intracellular K which is maintained by an active extrusion of Na ions; the action potential is the result of an inward movement of Na ions followed by an outward passage of K ions.

This paper deals with the effect of yohimbine on Na and K movements in resting tissue. In an earlier paper Shaw and Simon (1955) have criticised the rôle attributed to the Donnan potential and the Na pump in maintaining the elevated K level of the cell. Some of the results to be reported in this paper will also be used to support this criticism.

Methods.

The experimental details were similar to those described in an earlier paper (Shaw and Simon, 1955), i.e., one of a pair of sciatic nerves or sartorius muscles of the toad *Bufo marinus* was soaked in Ringer solution under varying conditions, the other member was treated identically except that different concentrations of yohimbine were added to the Ringer.

The extrusion of Na and the accumulation of K against a concentration gradient were brought about in a manner similar to that already described by Steinbach (1952) and the present author (1955), i.e., muscles and nerves were cooled in low-K Ringer and the alternation in ionic content on returning to warm, high-K Ringer determined.

In all experiments the tissues were tested for degree of yohimbinization by electrical stimulation. There were no changes in the volumes of the tissues under these experimental conditions.

Sufficiently large groups of muscles and nerves were taken to permit of statistical analysis. The mean and standard deviation (S.D.) were calculated from the logarithms of the observations as it was found that most of the results were not distributed normally. Corrections for the log mean and log S.D., as described by Sichel (1952), were made. The significance of the difference of means was tested by the "t" test. Where possible, individual pairs were compared, after taking logarithms, rather than group means.

RESULTS.

The effect of yohimbine on ion movements in excised muscle and nerve, when soaked in K-free Ringer.

As has been previously reported (for literature, see Shaw and Simon, 1955), there is a loss of K and a gain of Na when muscle is soaked in Ringer. As will be evident from Table 1, yohimbine has not affected these ionic movements. This was true even when yohimbine was used at a concentration sufficient to kill the tissue. Similar experiments were carried out with nerves. See Table 1.

The effect of yohimbine on ion movements in excised muscle and nerve, when soaked in K-free Ringer.

As yohimbine was found to be without effect on ion movements of tissues soaked in normal Ringer, we investigated its action on muscles and nerves which were submitted to the additional stress of K-free Ringer. It was found that soaking muscles in a large volume of K-free Ringer did not cause significant alteration of the ionic levels, as compared with muscles soaked in normal Ringer. Small K losses occurred in nerves soaked in K-free Ringer, but these could almost entirely be ascribed to loss of K from the extracellular space. See Table 2. The intracellular space was calculated as 50 p.c. of the total nerve, without regard to the distribution of solids, and K results have been corrected to account for this factor (Shaw and Simon, 1955).

When muscles soaked in K-free Ringer were compared with those soaked in K-free Ringer and yohimbine, it was found that there was no difference between the control and the treated tissue. See Table 3.

Potassium-free Ringer, plus yohimbine, brought about a slight loss of K from the nerve, but did not affect the Na level.

The effect of yohimbine on recovery movements of ions after cooling.

It has previously been shown (Shaw and Simon, 1955) that muscles and nerves gain Na and loose K to a greater extent at 3° C. than tissues held at room temperature. It was also found that these changes could be reversed by returning the tissue to room temperature in a potassium- and phosphate-enriched medium. The effect of yohimbine on this recovery process was studied.

Nerves and muscles were chilled to bring about K loss and Na gain, and then one of each pair was placed in the K-enriched Ringer containing yohimbine

TABLE I.

Effect of Yohimbine on Ionic Content.

Sartorius.

Concentration of Yohimbine	Time	Control	K mEq/Kg. Dry Wt.			Na mEq/Kg. Dry Wt.			Significance	Comments
			Yo.	Difference	Significance	Control	Yo.	Difference		
1 : 10^3	15 min.	345	337	−8	N.S.	264	264	0	N.S.	Moderately yohimbinised
1 : 10^4	5 hours									
1 : 10^3	2 hours	346	370	+24	N.S.	260	250	−10	N.S.	All muscles dead
3 : 10^4	2 hours	398	377	−21	N.S.	270	285	+15	N.S.	Well yohimbinised
2 : 10^3	4 hours	337	279	−58	N.S.	386	397	+11	N.S.	All muscles dead
					Sciatic					
5 : 10^4	18 hours	177	174	−3	N.S.	495	504	+9	N.S.	Well yohimbinised
1 : 10^3	18 hours	135	137	+2	N.S.	299	290	−9	N.S.	All nerves dead
3 : 10^3	1 hour	155	155	0	N.S.	343	362	+19	N.S.	All nerves dead
2 : 10^3	23 hours									

NOTE: In this and subsequent tables N.S. means not significant, S. means significant, i.e. probability between 0·05 and 0·01; V.S. means very significant, i.e. probability greater than 0·01.

TABLE 2.

The Effect of K-free Ringer on Ionic Content of Nerve and Muscle.

Time	K mEq/Kg. Dry Wt.		Difference	Significance	Na mEq/Kg. Dry Wt.		Difference	Significance
	Control	K-free			Control	K-free		
Sartorius 4 hours	341	342	+1	N.S.	325	319	−6	N.S.
Sciatic 22 hours	135	121	−14	N.S.	492	538	+46	N.S.

TABLE 3.

Effect of Yohimbine on Nerves and Muscles Soaked in K-free Ringer.

Concentration of Yohimbine	Time	K mEq/Kg. Dry Wt.			Signi-ficance	Na mEq/Kg. Dry Wt.			Signi-ficance	Comments
		Control	Yo.	Differ-ence		Control	Yo.	Differ-ence		
Sartorius 1 : 10^6 / 2 : 10^5	1 hour / 5 hours	255	251	−4	N.S.	184	193	+9	N.S.	Well yohimbinised
Sciatic 1 : 10^4	8 hours	103	89	−14	S.	306	313	+7	N.S.	Well yohimbinised

at 25° C. In an effort to ensure that the tissue was adequately yohimbinized before the "pumps" could function after warming, both control and the test tissue were soaked for half an hour at room temperature, after cooling, in 1/3 K Ringer plus yohimbine. The control was then weighed and analysed, and the test tissue placed in high-K Ringer plus yohimbine to permit the recovery processes to take place.

TABLE 4.

The Effect of Yohimbine on Recovery Movements of Na and K.

Concentration of Yohimbine	Time at 3° C	Time at 23° C	K gain mEq/Kg. Wet Wt.	Significance	Na loss mEq/Kg. Wet Wt.	Significance
Sciatic						
2×10^4	16 hrs.	4 hrs.	8·3	V.S.	6·5	V.S.
2×10^4	16 hrs.	4 hrs.	14·6	V.S.	0·3	N.S.
Sartorius						
$5 \times 10^{4*}$	16 hrs.	4 hrs.	−7·3	N.S.	+6·0	N.S.
2×10^4	16 hrs.	4 hrs.	20·4	V.S.	16·0	S.
2×10^4	6 hrs.	3 hrs.	12·5	S.	23·2	V.S.

* Yohimbine added to muscles at 3° C, after half an hour tissues transferred to (K × 3) Ringer plus yohimbine.

In all cases the passive increase in K, due to the high extracellular K in the "recovery" Ringer, has been deducted from the K gain. The results of these experiments may be seen in Table 4. It is obvious that the yohimbine has not prevented the extrusion of Na and reaccumulation of K, which takes place on returning the tissue to room temperature. The exception to this is the first sartorius experiment, when a high, and indeed lethal concentration of yohimbine was added to the muscle *in the cold*. This prevented the recovery process and brought about an additional (but not significant) K loss and Na gain.

DISCUSSION.

It is evident from Tables 1 and 3 that the passive movements of K and Na along their concentration gradients, which take place when the tissues are removed from the animal, are unaffected by yohimbine. This is so with muscle even when the gradient of K is increased by removing it from the external fluid. Nerve appears to be more sensitive to deprivation of K, and under these conditions yohimbine brought about slight K losses from the cell.

The ability of muscles and nerves to extrude Na against a concentration gradient has been demonstrated by experiments in which the tissues have been cooled and then rewarmed to room temperature in a K- and phosphate-enriched medium. At low temperature additional amounts of Na enter the cells and there is a concomitant loss of K. These movements are reversed when the tissue

is rewarmed. This reinstatement of the original ionic pattern requires energy, as the ions are moving against their concentration gradients. In the previous experiments it was shown that passive movements of Na and K were unaffected by yohimbine, now it has been demonstrated that active movements are also unaffected by this drug.

It would appear that the only exception to the statement that yohimbine does not influence the movements of ions in the resting state is provided by the finding that if almost lethal concentrations of yohimbine are added to the cold muscle then the reconstitution on warming does not take place. Under these conditions it seems that the metabolism of the cell is irreversibly depressed.

It is generally considered that this demonstration of the movements of ions against chemical gradients is evidence for the presence of at least one pump in the cell. In our previous publication we have given some reasons for an alternative explanation of this phenomenon (Shaw and Simon, 1955).

If the extrusion of Na is dependent upon a metabolic pump, then arrest of metabolism by whatever means might be expected to have a similar effect on Na and K movements. This is not the case. Treatment with iodoacetate and cyanide causes far greater ionic changes than mere cooling to 3° C. See Table 5. Ling (1952) points out that in cold muscle the ATP and CrP levels remain practically unchanged, indicating that their energy content has not been utilized. However, in the poisoned muscle the high energy phosphate compounds disappear. It is perhaps unwise to assume that constancy of CrP and ATP levels

TABLE 5.

Comparison of effect of low temperature and IAA (8mM/l) and Cyanide (24mM/l) on ionic content.

Time	K mEq/Kg. Dry Wt.		Na mEq/Kg. Dry Wt.	
	3° C	IAA and CN	3° C	IAA and CN
Sartorius				
2 hrs.	329	57·7	315	547
2 hrs.	357	77·4	254	599
4 hrs.	280	34·0	324	521
5 hrs.		18·0		647
6 hrs.	261		289	
6 hrs.	343		317	
10 hrs.	201		374	
16 hrs.	286		298	
24 hrs.	77		501	
Sciatic				
10 hrs.	134	68·3	468	613
12 hrs.	138		514	
16 hrs.	133	34·4	431	609
24 hrs.	124		545	

Each result is the mean of at least six observations.

indicate metabolic arrest. It is well known that muscle can twitch and develop heat at 0° C.

That the above mentioned similarity does not occur would seem to indicate that some other process is operative in maintaining the ionic imbalance. Shaw and Simon (1955) have put forward an adsorption postulate, whereby the K is retained in the cell by specific adsorption dependent on the integrity of the normal cell structure. The positioning of K within the cell requires energy, but its maintenance within the cell does not require a continuous supply of energy, as would be the case if a pump mechanism were operative.

The loss of K and gain of Na which takes place in the cold would be explained on our hypothesis as being due to the gradual destruction of adsorption sites, and the recovery movements on warming are due to a resynthesis of these sites.

As we have previously pointed out (Shaw and Simon, 1955), the reconstitution of the normal ionic content of cooled muscles on warming requires the presence of excess K and phosphate in the Ringer. The necessity for such enrichment could be explained as arising from one or both of two effects: (a) the raised K level, by a simple mass-action effect, increases the amount of K specifically adsorbed; (b) the well-known metabolic stimulatory action of K which may bring about an increased reconstitution of the adsorption sites. It is unlikely, however, that increasing the extracellular K threefold will have a marked effect on the cellular metabolism, as McIlwain (1952) found it necessary to increase the K level to 30 to 100 mM./l. to produce an increase in oxygen uptake.

The following facts require that one postulates two distinct mechanisms for the Na "pump" and Na "carrier." As it has been shown the movements of Na ions which take place in the resting cell are unaffected by yohimbine, this conclusion is supported by the fact that yohimbine is without action on the resting potential although, at the same time, the Na "pump" must be continuously in action (Holman, 1954). It has also been shown in this laboratory (MacKenzie, 1953) that the action of yohimbine resembles that of Na depletion; i.e., the effect of yohimbine on the passage of the nervous impulse resembles that of a Na-depleted Ringer and the yohimbine action is enhanced in a Na-poor solution. So it has been tentatively deduced that yohimbine interferes with the transport of Na during the active phase. Again confirmation of the suggestion that yohimbine acts only on active tissue is given by the discovery that yohimbine markedly depresses the action potential (Holman, 1954). Thus, as movements of Na ions in resting muscle are not affected by yohimbine while those in active muscle are, so one is forced to the conclusion that the Na "pump" is unaffected by yohimbine whilst the "carrier" is inhibited.

It has been shown (de la Lande, personal communication) that moderate concentrations of yohimbine, which produce the yohimbine effect in nerve, do

not affect the oxygen uptake. Higher concentrations have been found to depress the oxygen uptake by approximately 25 p.c. Doty and Gerard (1950) have also found yohimbine to be without effect on the resting oxygen uptake, but have shown it to depress the extra respiration associated with stimulation. This further emphasizes the selective action of yohimbine on the stimulated, rather than the resting tissue.

In conclusion, it may be said that yohimbine can serve as a useful tool in the investigation of those changes taking place when nerve or muscle is stimulated. Yohimbine (except in very high concentration) is without action on ionic movements in resting tissue, but does influence those of stimulated tissue, and it may be tentatively suggested that yohimbine is without action on the Na pump but inhibits the Na carrier mechanism. This hypothesis is only valid if one accepts the current view of the potential gradients in the living cell. However, evidence is presented in this paper which offers an alternative explanation.

SUMMARY.

When the sartorius muscle and the sciatic nerve of the toad are removed from the body and placed in Ringer solution, there tends to be a loss of K and a gain of Na. These ionic changes, which take place along concentration gradients, are unaffected by yohimbine.

When the excised tissues are soaked in K-free Ringer there is no additional gain of Na or loss of K. The presence of yohimbine does not influence the gain of Na but slightly increases the loss of K in nerves only.

In excised nerves and muscles which have been cooled to 3° C. the above ionic movements are enhanced; on warming ionic reconstitution takes place. The movements of the ions are now against concentration gradients. These movements are unaffected by yohimbine.

By use of the drug yohimbine it is possible to distinguish between movements of ions in the resting state and during stimulation; only during activity are ionic movements influenced by this drug. The mechanism of the Na "pump" of resting muscle is different from that of the "carrier" when the muscle is in the active state, in that the latter is affected by yohimbine and the former is not.

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A STUDY OF TWINS

BLOOD GROUPS AND OTHER DATA

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Determination of the ovular origin of human twins is always of parental interest and may be important in studying the susceptibility of genetically similar individuals to physical disorders (Burnet, 1953) and to psychotic and neurotic disturbances (Slater, 1953). In childhood and adult life, physical differences frequently permit recognition of the binovular nature of twins of the same sex, but such differences are usually not apparent in the neonatal period. Furthermore, the anatomy of placentae is sometimes difficult to interpret and is not always decisive. The primary purpose of the present work was to investigate the usefulness of blood groups in distinguishing uniovular from binovular twins. The opportunity was taken to compare differences in weights and lengths of the pairs according to the results of blood grouping, and material age and parity were also compared in the two groups.

MATERIALS AND METHODS.

During the period of this investigation, blood samples were obtained from all twins delivered at the Royal Hospital for Women, Sydney, and at the King George V Memorial Hospital for Mothers and Babies, Sydney. Two or three drops of blood were obtained from a needle prick of the heel of each subject and mixed with approximately 1·5 ml. of physiological saline solution. The red cells were washed twice with physiological saline solution before use and were tested within 24 hours of collection.

Tests were performed on every sample for the A_1A_2BO groups, the MNS system, and with anti-D, anti-C, anti-E and anti-c of the Rh system. Tests were usually made for the Fy(a) antigen and on some occasions for the P, Le(a), Lu(a) and Kell antigens. However, only the results of the ABO, MNS and Rh tests are considered in this paper. The results have not been included if one or both twins gave positive reactions with the direct Coombs test. The methods used are as described by Mourant (1952).

At the time of the blood-groups test the authors were not aware of the sex of the twins nor of their weight and length. This information, together with details of the maternal age and parity, was obtained later from the hospital records.

RESULTS.

Blood groups. The complete blood group results on 96 pairs of twins are shown in Table 4 and the essential features are summarized in Table 1.

The probability that any blood-group system will discriminate between binovular twins (or siblings) is dependent on the number of available antibodies in the system and on the corresponding gene frequencies in the population under test (Fisher, 1951). Without considering the sex of the subjects, it was found in the present series of 96 pairs that the ABO groups differed in 33 (34·4 p.c.), the MNS groups in 37 (38·5 p.c.), and the Rh groups in 32 (33·3 p.c.). Further, 36 (37·5 p.c.) were identical on all three systems, whilst 27 (28·1 p.c.) differed on one system only, 24 (25 p.c.) on two systems, and 9 (9·4 p.c.) on three systems.

TABLE 1.

The results of blood group tests on twins according to the blood group systems.

		Like sex		Unlike sex		Totals
		ABO alike	ABO unlike	ABO alike	ABO unlike	
Rh alike	MNS alike	29	6	7	2	44 } 64
	MNS unlike	5	7	5	3	20 }
Rh unlike	MNS alike	6	4	3	2	15 } 32
	MNS unlike	4	6	4	3	17 }
Totals		44	23	19	10	
		67		29		96

The gene frequencies were calculated for the three blood-group systems, considering, firstly, only the first twin of each pair and, secondly, considering the second twin of each pair.

The A_1A_2BO groups are analysed in Table 2. The gene frequencies have been calculated, using methods described by Race and Sanger (1950). In both series the difference between the observed and expected numbers is significant and it can be seen that this is due to an excess of group AB. No explanation can be offered for this finding. Bryce, Jakobowicz, McArthur and Penrose (1951) investigated the blood groups of 53 pairs of twins, but the frequencies of the ABO groups did not differ from those of Victorian blood donors. Boorman (1951) did not consider separately the 24 pairs of twins amongst her series of 2,000 consecutive infants, but found no disturbance of the ABO groups amongst the single births.

The percentage of group A subjects (36·3 p.c.) is almost exactly that of the adult donor population in New South Wales (de Burgh, Sanger and Walsh, 1946), but approximately half the subjects in both series were found to belong

TABLE 2.

Analysis of A_1A_2BO groups of twins.

Group	Observed absolute		Observed frequency		Expected absolute	
	Series I.*	Series II.**	Series I.	Series II.	Series I.	Series II.
O	46	48	0·4792	0·5000	50·27	49·89
A_1	19	19	0·1979	0·1979	20·76	19·74
A_2	15	15	0·1563	0·1563	16·40	15·59
B	6	8	0·0625	0·0833	6·55	8·32
A_1B	5	3	0·0521	0·0313	1·06	1·29
A_2B	5	3	0·0521	0·0313	0·96	1·17
Total	96	96	1·0001	1·0001	96·00	96·00

Gene frequencies

	Series I.	Series II.
O	= 0·7236	0·7209
A_1	= 0·1210	0·1163
A_2	= 0·1097	0·1050
B	= 0·0457	0·0578
χ^2	= 26·25	4·11
	(for 1 degree of freedom)	
P	<0·01	lies between 0·05 and 0·02

* The first of each pair of twins is considered in Series I.
** The second of each pair of twins is considered in Series II.

to subgroup A_2. This is a considerably greater percentage than is usually found. The same high incidence of group A_2 was observed by Witebsky and Engasser (1949) when testing 34 newborn infants belonging to group A. They observed that about half their subjects gave reactions with anti-A serum which were similar to or weaker than the control adult group A_2 cells, whilst the remainder reacted more strongly than the group A_2 cells, but usually more weakly than adult group A_1 cells. They stressed the difficulty of classifying cells of the newborn into sharply defined subgroups. The phenomenon is unlikely to be due in the present series to weak anti-A_1 serum (prepared by absorbing immune group B serum with group A_2 cells) because the same serum was used for testing several hundred blood samples from Pacific natives with positive results in every instance. ABO group differences amongst twin pairs have depended in three instances on the finding of group A_1 in one twin and of group A_2 in the other; but in only one of these three was the determination of binovularity based exclusively on the ABO groups.

The MNS gene frequencies are similar in both series of twins, and the expected phenotype numbers, as calculated from the gene frequencies, do not differ significantly from the observed numbers.

An analysis of the Rh system is shown in Table 3. The gene frequencies

TABLE 3.

Analysis of the Rh groups of twins.

Reaction with anti sera				Most common genotypes	Observed absolute		Observed frequency		Expected absolute	
C	c	D	E		Series I.*	Series II.**	Series I.	Series II.	Series I.	Series II.
+	−	+	−	CDe/CDe	15	21	0·1563	0·2188	15·00	21·01
+	+	+	−	CDe/cde	32	32	0·3333	0·3333	35·34	38·89
+	+	+	+	CDe/cDE	22	19	0·2292	0·1979	9·56	8·91
−	+	+	+	cDE/cDE or cDE/cde	4	6	0·0417	0·0625	13·09	9·19
−	+	−	−	cde/cde	22	18	0·2292	0·1875	22·00	18·00
+	+	−	−	Cde/cde	1	0	0·0104	0·0000	1·00	0·00
				Total	96	96	1·0001	1·0001	95·99	96·00

Gene frequencies

	Series I.	Series II.
CDe	= 0·3845	0·4678
cDE	= 0·1259	0·0992
cde	= 0·4787	0·4330
Cde	= 0·0109	0·0000
χ^2	= 22·82	13·75
	(for two degrees of freedom)	
P	<0·01	<0·01

* The first of each pair of twins is considered in Series I.
** The second of each pair of twins is considered in Series II.

were calculated by standard methods described by Race and Sanger (1950) and in both series the observed results differ significantly from the expected. It is apparent from inspection of the Table that this divergence is due to an excess of subjects giving the reactions (+ + + +), of which the commonest genotype is CDe/cDE (R_1R_2) and to a deficiency of subjects giving the reactions (− + + +), of which the commonest genotypes are cDE/cDE (R_2R_2) and cDE/cde (R_2r). These findings are again unlikely to be due to the use of weak anti-E sera because the sera were always well controlled, because consistent results were obtained in other series of blood samples, and because false negative reactions would be expected in both types of reactions, (+ + + +) and (− + + +). No adequate explanation can be offered for the results presented above. As far as the authors are aware, there have been no published results of the Rh genotypes of newborn infants; but it is well known to blood group serologists that false positive reactions with anti-E are sometimes observed in infants suffering from haemolytic disease of the newborn. The results presented in this paper suggest the necessity for more complete investigation of the Rh groups of newborn infants.

TABLE 4.

I. *Sex alike, ABO alike, MNS alike, Rh alike* (29 pairs)

1.	O	MS	CDe/CDe	16.	A_1	Ms	CDe/cde
2.	O	MS	CDe/CDe	17.	A_1	MNS	CDe/cde
3.	O	MS	CDe/cde	18.	A_1	MNS	CDe/cde
4.	O	MNS	CDe/CDe	19.	A_1	Ns	CDe/CDe
5.	O	MNS	CDe/cDE	20.	A_2	MS	CDe/cDE
6.	O	MNS	CDe/cDE	21.	A_2	Ms	CDe/cde
7.	O	MsNs	CDe/cde	22.	A_2	Ms	CDe/cde
8.	O	MsNs	CDe/cde	23.	A_2	MNS	CDe/CDe
9.	O	MsNs	CDe/cDE	24.	A_2	MNS	CDe/cde
10.	O	Ns	CDe/CDe	25.	A_2	MsNs	CDe/CDe
11.	O	Ns	CDe/cde	26.	A_2	NS	CDe/cde
12.	O	Ns	CDe/cDE	27.	B	MsNs	CDe/CDe
13.	A_1	MS	CDe/cDE	28.	A_1B	MNS	CDe/CDe
14.	A_1	MS	cde/cde	29.	A_2B	MsNs	cde/cde
15.	A_1	MS	cde/cde				

II. *Sex alike, ABO alike, MNS unlike, Rh alike* (5 pairs)

30.	O	MNS	CDe/cDE	33.	A_1	MNS	CDe/cde
	O	MS	CDe/cDE		A_1	MS	CDe/cde
31.	O	MNS	CDe/CDe	34.	A_1	MNS	CDe/cde
	O	Ns	CDe/CDe		A_1	MsNs	CDe/cde
32.	O	Ns	CDe/cde				
	O	MNS	CDe/cde				

III. *Sex alike, ABO alike, MNS alike, Rh unlike* (6 pairs)

35.	O	MsNs	CDe/cde	38.	O	MsNs	cDE/cde
	O	MsNs	cDE/cde		O	MsNs	cde/cde
36.	O	MsNs	CDe/cde	39.	O	NS	CDe/cDE
	O	MsNs	CDe/cDE		O	NS	CDe/CDe
37.	O	MsNs	CDe/cDE	40.	A_2	MsNs	cde/cde
	O	MsNs	cde/cde		A_2	MsNs	CDe/cde

IV. *Sex alike, ABO alike, MNS unlike, Rh unlike* (4 pairs)

41.	O	MS	CDe/CDe	43.	O	Ns	Cde/cde
	O	MNS	CDe/cde		O	MsNs	CDe/cde
42.	O	MNS	CDe/cde	44.	A_2B	MsNs	cde/cde
	O	Ns	CDe/CDe		A_2B	MNS	CDe/cde

V. *Sex alike, ABO unlike, MNS alike, Rh alike* (6 pairs)

45.	O	MsNs	CDe/cde	48.	A_1	Ns	cDE/cde
	A_1	MsNs	CDe/cde		A_2	Ns	cDE/cde
46.	O	MNS	CDe/cDE	49.	A_2	Ns	cde/cde
	A_2	MNS	CDe/cDE		O	Ns	cde/cde
47.	A_1	Ns	CDe/cDE	50.	A_1B	MsNs	CDe/cde
	O	Ns	CDe/cDE		A_1	MsNs	CDe/cde

TABLE 4—*continued.*

VI. *Sex alike, ABO unlike, MNS unlike, Rh alike* (7 pairs)

51.	O	Ns	CDe/CDe	55.	A_1	MsNs	CDe/CDe
	A	NS	CDe/CDe		A_2	MS	CDe/CDe
52.	O	MsNs	cde/cde	56.	A_2	Ms	CDe/cDE
	B	MNS	cde/cde		O	NS	CDe/cDE
53.	O	Ns	CDe/cde	57.	A_1B	MNS	CDe/cDE
	B	MNS	CDe/cde		A_1	MS	CDe/cDE
54.	A_1	MS	CDe/cDE				
	O	MNS	CDe/cDE				

VII. *Sex alike, ABO unlike, MNS alike, Rh unlike* (4 pairs)

58.	O	MNS	CDe/cDE	60.	B	MNS	cde/cde
	A_1	MNS	CDe/CDe		O	MNS	CDe/cde
59.	A_1	MsNs	CDe/cde	61.	A_2B	Ns	CDe/cde
	A_1B	MsNs	cDE/cde		B	Ns	CDe/CDe

VIII. *Sex alike, ABO unlike, MNS unlike, Rh unlike* (6 pairs)

62.	O	MS	cde/cde	65.	A_1	Ms	CDe/cde
	A_2	NS	cDE/cde		O	MNS	cde/cde
63.	O	Ns	CDe/cde	66.	A_1	MNS	cde/cde
	A_2	MNS	cde/cde		O	MS	CDe/cDE
64.	O	Ns	cde/cde	67.	A_2	MS	cde/cde
	B	MNS	CDe/cde		O	MsNs	CDe/cDE

IX. *Sex unlike, ABO alike, MNS alike, Rh alike* (7 pairs)

68.	O	MS	CDe/cde	72.	O	MsNs	CDe/CDe
69.	O	MS	CDe/cDE	73.	A_1	MS	cde/cde
70.	O	MS	cde/cde	74.	A_2B	NS	cde/cde
71.	O	Ms	cDE/cde				

X. *Sex unlike, ABO alike, MNS unlike, Rh alike* (5 pairs)

75.	O	MsNs	CDe/cDE	78.	A_2	NS	CDe/cDE
	O	MNS	CDe/cDE		A_2	Ns	CDe/cDE
76.	O	MsNs	CDe/cde	79.	B	Ns	cde/cde
	O	Ns	CDe/cde		B	Ms	cde/cde
77.	A_1	NS	cde/cde				
	A_1	MsNs	cde/cde				

XI. *Sex unlike, ABO alike, MNS alike, Rh unlike* (3 pairs)

80.	O	MS	CDe/cDE	82.	A_1B	MS	CDe/cDE
	O	MS	CDe/CDe		A_1B	MS	CDe/cde
81.	O	NS	cde/cde				
	O	NS	CDe/cde				

TABLE 4—*continued.*

XII. *Sex unlike, ABO alike, MNS unlike, Rh unlike* (4 pairs)

83.	O	MS	CDe/cDE	85.	O	MNS	cde/cde
	O	MsNs	CDe/CDe		O	MS	cDE/cDE
84.	O	Ms	CDe/cde	86.	A_2	MNS	CDe/cDE
	O	MS	CDe/cDE		A_2	MS	CDe/cde

XIII. *Sex unlike, ABO unlike, MNS alike, Rh alike* (2 pairs)

87.	B	MsNs	CDe/cde	88.	A_1B	MN	cde/cde
	O	MsNs	CDe/cde		O	MN	cde/cde

XIV. *Sex unlike, ABO unlike, MNS unlike, Rh alike* (3 pairs)

89.	O	MNS	CDe/CDe	91.	B	MsNs	CDe/cde
	B	MS	CDe/CDe		O	Ms	CDe/cde
90.	A_2	MNS	CDe/cde				
	A_1	Ns	CDe/cde				

XV. *Sex unlike, ABO unlike, MNS alike, Rh unlike* (2 pairs)

92.	A_2	MNS	cde/cde	93.	A_2B	MS	CDe/cde
	O	MNS	CDe/CDe		B	MS	cde/cde

XVI. *Sex unlike, ABO unlike, MNS unlike, Rh unlike* (3 pairs)

94.	O	MsNs	cde/cde	96.	B	Ms	cDE/cde
	A_1	MS	CDe/cde		A_1	MsNs	cde/cde
95.	A_1	Ms	CDe/cde				
	O	MNS	cde/cde				

Maternal age and parity.

It has been stated that the frequency of binovular twinning is considerably greater at the higher maternal ages and parities but that uniovular twinning occurs with approximately the same frequency in all maternal age groups (McArthur, 1953). The present series was analysed according to maternal age in two groups—those pairs which had been distinguished either by sex or by blood groups, and those pairs which were not distinguished by either means. It was found that 50·5 p.c. of all the mothers were less than 30 years of age, but that there was no difference in age distribution between the two series ($\chi^2=3\cdot66$ for 5 degrees of freedom).

A similar analysis was made in regard to the parity of the mother. Again no difference was detected between the two series ($\chi^2=3\cdot41$ for 7 degrees of freedom).

Weight. An analysis of the differences in weight between the two members of each pair has been made for the following groups:

A Like sex, like blood groups
B Like sex, unlike blood groups
C Unlike sex, like blood groups
D Unlike sex, unlike blood groups

The mean squares of the differences in weights of the different groups were compared and tested by means of the usual variance ratio tests. The following ratios were obtained:

B/A=1·5920	n_1, 38; n_2, 29
D/C=1·3443	n_1, 22; n_2, 7
A/C=1·5785	n_1, 29; n_2, 7
B/D=1·8693	n_1, 38; n_2, 22
A/D=1·1676	n_1, 29; n_2, 22
B/C=2·5130	n_1, 38; n_2, 7

None is significant at the 5 p.c. level. The mean weight of all infants was 88 ounces.

Length. A similar analysis was undertaken of the differences in lengths between the two members of each pair, but again the variance ratios were not significant. The mean length of all infants was 19 inches.

Sex. Amongst the 67 pairs of like-sexed twins, 30 represented males and 37 females. This gives a sex ratio of ·864 males to one female, a ratio which does not differ significantly from 1 to 1.

DISCUSSION.

In this work four distinguishing factors, each on a different chromosome, have been used, viz., sex, ABO group, Rh group and MNS group. Without reference to the others, each factor has distinguished a number of binovular twins, and in the series each factor has been of approximately the same value. On the average, a single factor has distinguished 34 p.c. of the pairs examined. Six different combinations of any two of these factors can be obtained, distinguishing on an average 53 p.c. of the pairs. The four combinations of three factors distinguish on an average 64 p.c., whilst all four factors together distinguish 70 p.c. These figures are plotted in Fig. 1, in which the curve is almost exactly the arc of a circle.

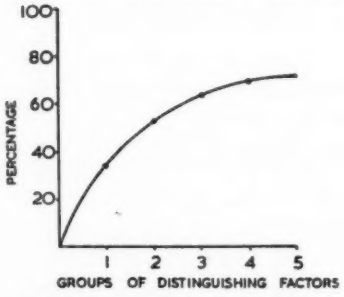

Fig. 1. Four factors were used to distinguish the binovular twins in the series investigated—sex, ABO group, MNS group and Rh group. The average percentages distinguished by a single factor, by two factors, and so on, when plotted on the graph give a curve which is the arc of a circle.

In any investigation, the percentage of twins not distinguished can be calculated from the formula

$$N=U+B\ (1-p_1)\ (1-p_2)\ \ldots\ \ldots\ (1-p_n)$$

where U is the percentage of uniovular pairs, B the percentage of binovular pairs, and p_1, p_2, etc., the probability that any system employed will distinguish

binovular twins. The values for p can be calculated for any blood group system. It follows from the above formula that as the number of distinguishing tests is increased, the number of binovular twins not discriminated will become progressively less. An inspection of the graph drawn for the present series shows that the four factors used have distinguished the great majority of binovular pairs, but that one further test of comparable discriminating power would be necessary to approximate the maximum.

Many workers have confirmed Weinberg's differential method of assessing the number of uniovular pairs in a series of twins. This is based on the assumption that, among binovular twins, there must be an equal number of like sex and of unlike sex pairs. In the present series 29 pairs were of unlike sex, and, according to Weinberg's formula, there should be a similar number of binovular pairs of like sex. The residual 38 pairs should be uniovular. Actually only 29 pairs were not distinguished by either sex or blood groups, but this difference is not significant.

It might be contended that the unusual findings with the subgroups of A and with anti-E, limit the use of these systems in the newborn. However, the factors which cause the anomalous results would presumably be operative in both twins of a uniovular pair, and possibly in either or both of a binovular pair. They are, therefore, more likely to prevent discrimination than to produce false distinctions.

SUMMARY.

The ABO, MNS and Rh blood groups of 96 pairs of twins were determined to investigate the value of these tests in distinguishing the binovular pairs. The percentage of binovular pairs established (1) by sex alone was 30 p.c.; (2) by sex and ABO group was 54 p.c.; (3) by sex, ABO group and Rh group was 65 p.c.; and (4) by sex, ABO group, Rh group and MNS group was 72 p.c. From theoretical considerations it is probable that the four factors used together distinguish almost all the binovular pairs.

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INHIBITION BY BORATE OF AMMONIA FORMATION AND GLUTAMINE SYNTHESIS IN BRAIN TISSUE

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INTRODUCTION.

It was shown in an earlier publication that borate inhibits the respiration of brain tissue incubated in plain phosphate Ringer solution, but has little effect on the oxygen uptake in the presence of glucose (Trautner and Messer, 1953). Glucose, however, neither prevents nor abolishes the symptoms of borate poisoning *in vivo* (Pfeiffer, Hallman and Gersh, 1945), and it appears that the site of the toxic effects of borate poisoning would have to be sought elsewhere. The investigation was therefore extended to the nitrogen metabolism of brain. This report deals with the action of borate on (a) ammonia formation and (b) glutamine synthesis in guinea-pig cerebral cells incubated in phosphate Ringer solution.

METHODS AND MATERIALS.

Guinea-pig cerebral tissue was used as a finely-dispersed mince, as described in a previous paper (Trautner and Messer, 1953). Oxygen uptake was measured by the Warburg technique, using flasks with single side-arms and of 25–30 ml. capacity. Standard conditions of incubation were: temperature, 37·5° C.; weight of fresh tissue per flask, 120 mg., suspended in 3 ml. phosphate Ringer solution of pH 7·4 (Krebs, 1933); gas phase, air or nitrogen; rate of shaking, 100 per minute.

In experiments where the medium contained borate initially, a calculated amount of the sodium chloride in the Ringer solution was replaced by the required quantity of sodium borate to preserve the isotonicity of the medium; the pH was readjusted to 7·4 with HCl. In experiments where it was required to add borate during the course of incubation, neutralized sodium borate solution was tipped in from the side-arm: this made the medium slightly hypertonic, but control experiments with equivalent quantities of sodium chloride showed that the increase in tonicity did not affect the respiration or ammonia formation of the tissue. All borate concentrations are expressed in terms of H_3BO_3.

Ammonia was determined by the microdiffusion method of Conway (1947). Glutamine was estimated as amide-NH_3 by conversion of its amide-N to ammonia by acid hydrolysis, using the method described by Krebs (1935); the amide-NH_3 was given by the difference between the amounts of ammonia found before and after hydrolysis.

Since it was observed by Gilbert, Price and Greenstein (1949) that the non-enzymic desamidation of glutamine is slightly accelerated by borate, it was deemed desirable to deter-

mine whether this effect was of significance for the present investigation. To this end, glutamine was incubated in phosphate Ringer at 37·5° C. for 3 hours with and without 0·13 M borate, and the solution then analysed for ammonia. The amounts of ammonia found in each case were negligible, showing that no significent desamidation of glutamine had occurred in the presence or absence of borate.

The glutamine (97 p.c. pure) used in these experiments was kindly supplied by Dr. F. J. R. Hird, of the Department of Biochemistry of this University. All other materials were of reagent quality.

RESULTS.

Ammonia formation.

When brain tissue is incubated in the absence of glucose, relatively large amounts of ammonia are liberated into the medium (Weil-Malherbe, 1936; see also Dickens and Greville, 1933). The time-course of this ammonia formation as observed under the conditions of our experiments is illustrated in Fig. 1, together with the oxygen consumption of the tissue and the effect of 0·13 M borate on both processes. The molar ratios at various times of oxygen consumed to ammonia formed are presented in Table 1.

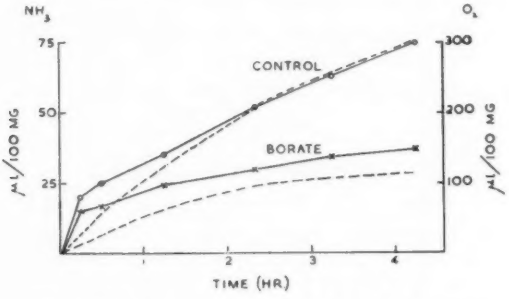

Fig. 1. Effect of 0·13 M borate on ammonia formation and oxygen uptake of brain tissue incubated in plain phosphate Ringer solution.

Broken lines: ammonia formation.

Solid lines: oxygen uptake.

Ammonia formation corrected for preformed ammonia (average 3·5 µl./100 mg. tissue).

Timed from moment flasks were placed in bath; oxygen consumption during 15-min. equilibration period by extrapolation.

It is seen (Fig. 1) that *in the absence of borate* there was a rapid initial liberation of ammonia lasting about 15 minutes, followed by a slower and more constant, slightly declining rate. The shape of the curve resembled that of the oxygen uptake, but the initial oxygen uptake was less marked than the initial ammonia formation, so that the ratio of oxygen consumption to ammonia forma-

TABLE 1.

Effect of borate on the mean ratio: $\frac{\textit{moles oxygen consumed}}{\textit{moles ammonia formed}}$

(Conditions as in Fig. 1.)

Time (min.)	No. of Detmns.	Ratio	
		Control	Borate
15	3	1·2	0·9
30	2	2·2	1·5
75	4	3·5	2·7
140	6	3·9	3·1
195	3	4·1	3·2
255	5	4·0	3·1

tion increased rapidly during the first 75 minutes from about 1 to 4; after this time it tended to remain constant at a value of about 4·0 (Table 1).

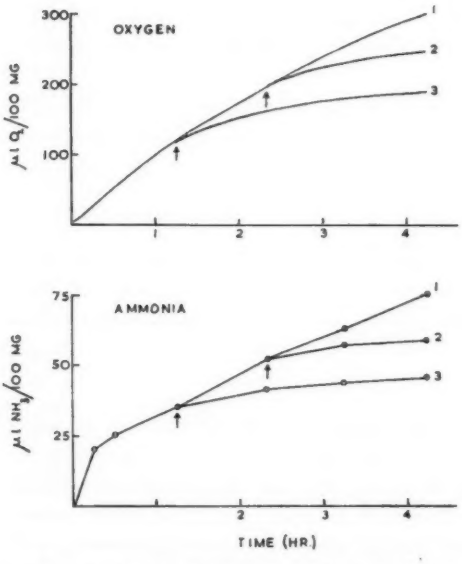

Fig. 2. Effect of delayed addition of 0·13 M borate on ammonia formation and oxygen uptake. Conditions as in Fig. 1.

Curve 1: no borate.

Curve 2: borate added after 150 min.

Curve 3: borate added after 95 min.

In the presence of 0·13 M borate, both the ammonia formation and the oxygen uptake were depressed (Fig. 1), but the overall inhibiting effect on the oxygen uptake was greater, largely because the initial ammonia formation appeared to be relatively resistant to borate. Later, after about 75 minutes of incubation, the ammonia formation was inhibited to about the same extent as the oxygen uptake, the ratio of oxygen consumption to ammonia formation remaining constant at a value of just over 3 (Table 1).

In another series of experiments, borate was added to the medium at various times after the start of incubation. Under these conditions the depressant effect of the drug on the oxygen uptake and ammonia formation appeared relatively rapidly, no lag period similar to that found with early addition being ob-

served (Fig. 2). Both processes were inhibited to about the same extent, the ratio of oxygen consumption to ammonia formation remaining constant at a value of about 4·0, both in the presence and absence of borate.

With lower concentrations of borate these effects were less pronounced; the lowest concentration found to have an effect within 5 hours of incubation was of the order of 0·05 M. At all effective concentrations of borate the relation between the effect on the oxygen uptake and ammonia formation was essentially the same as that found with 0·13 M borate.

When the tissue was incubated in an atmosphere of nitrogen instead of air, nearly all the ammonia formation occurred during the first 75 minutes, after which it practically ceased. The total ammonia formed in 255 minutes amounted to about one-third of that formed during the same time in the presence of oxygen (Table 2). Borate had no effect on the ammonia formation under these conditions.

TABLE 2.

Ammonia formation in the absence of oxygen

Gas phase, nitrogen. (Average values for two experiments.)

Time (min.)	Ammonia Formed (μl/100 mg. tissue)	
	Control	Borate
30	15	13
75	20	20
140	21	22
255	23	22

It may be noted that the above experiments were all carried out in plain phosphate Ringer solution. When the medium contained glucose, relatively little ammonia formation occurred (see also Krebs, 1935), and borate did not reduce this any further.

Glutamine synthesis.

The synthesis of glutamine by brain tissue was first investigated in detail by Krebs (1935), who found that the presence of glucose was essential for this process. Weil-Malherbe (1936), however, observed that significant synthesis occurred also in the presence of pyruvate or of L-glutamate or α-ketoglutarate. In the present experiments it was found that maximal glutamine synthesis occurred only in the presence of both glucose (0·01 M) and L-glutamate (0·02 M), as well as of free ammonia (in the form of ammonium chloride). When glutamate was omitted or substituted by α-ketoglutarate, or when glucose was omitted or substituted by pyruvate, relatively little glutamine synthesis occurred.

TABLE 3.

Effect of borate on glutamine synthesis by brain tissue.

Glucose, 0·01 M; L-Glutamate, 0·02 M; Borate, 0·13 M.
Ammonia, 56 μl/100 mg. tissue (as ammonium chloride).
Time of incubation, 180 min.
Values expressed as μl/100 mg. tissue.

	Free NH_3	Total NH_3	Amide-NH_3	$\frac{\text{Amide-}NH_3 \times 100}{\text{Total } NH_3}$	Oxygen Uptake
Control	21	95	74	78	370
Borate	78	82	4	5	361

Table 3 presents the results of a typical experiment, where with a three-hour period of incubation and *in the absence of borate*, little free ammonia and relatively large amounts of glutamine were found in the medium at the end of the experiment. *In the presence of 0·13 M borate*, however, no glutamine was formed, almost all the ammonia being in the free form. The percentage of the total ammonia present as amide ammonia thus fell from 78 in the absence of borate to 5 in its presence. There was also some slight reduction in the total ammonia in the presence of borate. It may be noted that, since glucose was present, the borate had almost no effect on the oxygen uptake (Trautner and Messer, 1953).

When lower concentrations of borate were used, the inhibiting effect on glutamine synthesis was less marked; the effect disappeared at concentrations of about 0·03 M (Table 4).

TABLE 4.

Inhibition of glutamine synthesis at various concentrations of borate.

(Conditions as in Table 3.)

Borate concentration (M)	$\frac{\text{Amide-}NH_3 \times 100}{\text{Total } NH_3}$
0·13	7
0·10	20
0·06	54
0·03	75
0	81

Experiments were carried out to determine whether the inhibiting effect of borate on glutamine synthesis could be counteracted by increasing the concentrations in the medium of glucose, glutamate, or magnesium ion. No such effect was observed. It was found, however, that in the absence of borate, high concentrations of magnesium (0·012 M) slightly inhibited glutamine synthesis.

DISCUSSION.

The results presented show that borate inhibits ammonia formation as well as glutamine synthesis in brain tissue. These effects may be of interest in connection with borate poisoning, a condition which has occurred occasionally as a result of treatment of eczema with boric acid powder or after accidental ingestion of borate (MacGillivray and Fraser, 1953). The drug appears to affect particularly the central nervous system, prominent symptoms being convulsions and coma; post-mortem examinations in fatal cases have shown that large amounts of boric acid, ranging from 50 to 300 mg. p.c., accumulate in the brain, particularly in the grey matter (McNally and Rust, 1928; Pfeiffer *et al.*, 1945). In the present experiments the smallest concentration found to have a significant effect within the experimental period of about 4 hours was approximately 0·04 M, corresponding to 248 mg. p.c.

The interpretation of the results and of their significance is limited by the fact that neither the mechanism of ammonia formation nor the rôle of ammonia and glutamine in brain are yet fully understood.

The source of the ammonia formed in brain preparations is still unknown. The question has recently been discussed by Weil-Malherbe (1953), who suggests that one part of the ammonia formation is autolytic, while another part depends on the integrity of structural units and is linked with oxidative phosphorylation. The present results confirm that, in the absence of glucose, part at least of the ammonia formed is connected with an oxidative process which is inhibited by borate; to what extent oxidative phosphorylation may take place under these conditions must remain doubtful. The rapid initial ammonia formation shown by our preparation appeared to be relatively resistant to borate as well as independent of oxygen supply. It may be autolytic, but is not necessarily non-oxidative, owing to the presence and possible rôle of endogenous hydrogen acceptors (Dickens and Greville, 1933).

Glutamine synthesis in brain has been interpreted by Weil-Malherbe (1950) as a means of detoxicating ammonia, but the process may have wider significance in view of the fact that glutamine is a constituent of proteins (see review by Waelsch, 1952a). Furthermore, the discovery in mammalian tissues of enzyme systems, which catalyze the transfer of the glutamyl moiety of glutamine to hydroxylamine (Schou, Grossowicz, Lajtha and Waelsch, 1951) and of γ-glutamyl peptides to amino-acids (Hanes, Hird and Isherwood, 1952), has led to the hypothesis that glutamine may play a rôle in peptide or protein synthesis, acting as a substrate "through which the energy of the energy-rich phosphate is channelled into the formation of peptide bonds" (Waelsch, 1952b). The hypothesis, which is still under discussion (Hendler and Greenberg, 1954), suggests that glutamine synthesis may be viewed as a process of ammonia utilization as well as detoxication. Conceivably, therefore, both the formation of excess ammonia

in the brain as well as interference with protein metabolism may play a rôle in borate poisoning. The observation that the symptoms of borate poisoning take some time to appear, but persist for several weeks after the use of the drug has been discontinued (MacGillivray and Fraser, 1953) would be consistent with the hypothesis of damage to proteins or protein-replacing mechanisms. It may be noted, furthermore, that chronic administration of borate to immature rats has been shown to inhibit growth (Pfeiffer, *et al.*, 1945). In the absence of further evidence, however, any interpretation must necessarily be tentative.

Nothing very definite can be said concerning the mechanism by which borate inhibits glutamine synthesis. Interference with the utilization of adenosine triphosphate (ATP), which substance is known to be required for glutamine synthesis as a source of energy (Speck, 1947; Elliott, 1948), could occur by virtue of the fact that borate forms a complex with adenylic acid (AMP) by combination with the ribose moiety (Klimek and Parnas, 1932). A similar complex may be formed with ATP; but even if this were not the case, formation of the AMP-borate complex could interfere with the phosphorylation of AMP, thus displacing the normal balance between the adenosine phosphates and lowering the concentration of available ATP. Whether the extent of these effects would be sufficiently great to inhibit glutamine synthesis is admittedly an open question. Further investigations, using an enzyme system such as that employed by Elliott (1948), would probably provide a partial answer.

SUMMARY.

The effect of borate on ammonia formation and glutamine synthesis of guinea-pig cerebral tissue incubated in phosphate Ringer solution was investigated.

Borate in 0·13 M concentration markedly depressed ammonia formation of cerebral tissue incubated in the absence of added oxidisable substrate; the extent of this effect was similar to that of the depressant action of borate on the oxygen uptake. Lower concentrations of borate had less effect, none being observed with concentrations below 0·05 M.

Borate in 0·13 M concentration completely inhibited glutamine synthesis in the presence of glucose, L-glutamate and added ammonia. The lowest concentration of borate which had an inhibiting effect was about 0·03 M. The effect could not be reversed by increasing the concentrations in the medium of glucose, L-glutamate or magnesium.

The possible significance and mechanism of these effects is tentatively discussed.

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NEW TEST ANIMAL FOR ACETYLCHOLINE ASSAY

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(Accepted for publication 1st October, 1954.)

It was first indicated by Smith and Levin (1938) that the heart of *Venus mercenaria* could be used as a test object for acetylcholine assays since it had been shown by Prosser and Prosser (1937) that the inhibitory nerves of the *V. mercenaria* heart act by liberating acetylcholine, to which the heart is extremely sensitive.

Having the need for a reliable and fairly sensitive assay method for acetylcholine, we selected the *V. mercenaria* method as being the most suitable. Although this species is not available in Australia, Jullien (1936) has shown that the hearts of a number of molluses are inhibited by acetylcholine. Therefore, a number of locally available molluses were examined, including *Pinna menkei,* the hammer oyster; *Notovola fumatus,* the scallop; *Anadara trapezia,* the Sydney cockle; *Plebidonaz deltoides,* the beach pippi; *Tapes turgida,* the tapestry cockle; *Circe sugillata,* the round tapestry shell; and *Hydatina physa,* the bubble shell. Of these only *Tapes turgida* showed any promise and further studies were confined to this species.

Tapes turgida is a lamellibranch mollusc belonging to the same family as *Venus mercenaria,* the family Veneridae. It is slightly smaller than *Venus,* only growing to about three inches on its longest axis. An estuarine form, *T. turgida* is found at or just below low tide-mark on mud-flats. Usually, specimens may be collected from the surface of the flats, but the majority lie buried two or three inches deep in the mud. Their numbers fluctuate seasonally so that they are readily obtainable in fairly large numbers in late spring and summer, but may be very difficult to find in autumn. It has been suggested that this fluctuation is due to migration into deeper water.

An investigation of the pharmacological responses and behaviour of the isolated ventricle of *T. turgida* was carried out, adhering fairly closely to the methods used on *V. mercenaria.* The results of the investigation are reported briefly below.

METHOD.

For the isolation of the *Tapes* ventricle, we have adopted a rather different technique to that suggested by Wait (1943) and Welsh and Taub (1948) for *Venus.* The valves of

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the shell are separated by inserting a thin steel spatula between them at the posterior end and scraping the attachments of the two adductor muscles and the mantle from the uppermost valve. The shell is then only held shut by the interlocking-hinge teeth and the elastic ligament which are readily parted by applying a little pressure. With the shell opened, the pericardial sac is seen to be covered by the thin mantle and gills of that side. Cutting the dorsal attachments of these and pulling them aside reveals the distended pericardial sac in which the heart may be seen beating slowly. The thin-walled sac is then cut away revealing the heart, as shown in Fig. 1, and threads are attached at each end of the ventricle as indicated in Fig. 1a.

NEW TEST ANIMAL FOR ACETYLCHOLINE ASSAY

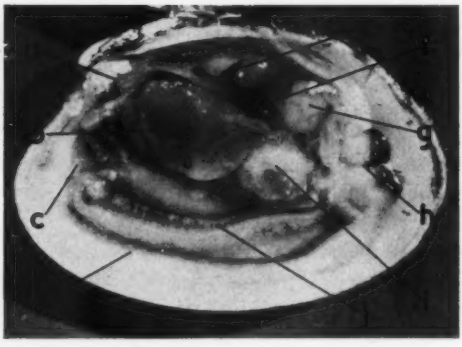

Fig. 1. *Tapes turgida* with left valve removed, the left mantle and gills cut away and the pericardium opened.
a. Hinge teeth.
b. Gill overlying visceral mass.
c. Anterior adductor muscle.
d. Right mantle fringe.
e. Pericardial cavity and contents.
f. Kidney.
g. Posterior adductor muscle.
h. Excurrent and incurrent siphons.
i. Pallial muscle.
j. Fringe of left mantle.

Fig. 1a. Enlarged view of pericardial region showing location of ties.
a. Anterior tie.
b. Ventricle.
c. Posterior tie.
d. Aortic bulb.
e. Kidney.
f. Left atrium.

The above ties include the rectum which runs directly through the ventricle. This means that any movement of the rectum is also recorded, which probably explains the periodic change in the baseline occasionally seen. Such fluctuation is not common, however, being seen in less than 5 p.c. of preparations, and does not seem to necessitate the use of atrioventricular rather than aortic ties. The atrioventricular ties have not been used because the two atria are situated close together on the lower surface of the ventricle, and would therefore presumably not transmit the contractile force of the whole ventricle, but only that portion stretched between them from the one side. Another reason is the fragility of the atrial walls compared with the anterior and posterior aortae.

It has been found advisable to make the anterior tie around the ventricular muscle rather than the dense capsule of the visceral mass which it adjoins. Isolation is completed

by cutting through the anterior and posterior aortae and atrioventricular junctions.

Undue handling and stretching of the ventricle are to be avoided as these tend to damage it.

The ventricle is set up in much the same manner as described by Wait (1943). A 10 ml. bath is used, in which the fluid can be changed by filling from the bottom and allowing the excess to escape through an overflow tube. The bath should not be emptied while the ventricle is in it as this is deleterious to the preparation. This organ bath is set up in a large water bath, the temperature of which is regulated by recirculating the bath fluid through a refrigerating unit and making finer adjustments by means of a heater immersed in the bath and thermostatically controlled. This combination allows thermo-regulation from below zero to 40° C.

Aerated sea-water is quite a satisfactory medium as was found by Prosser (1940). 0·25 gm. of glucose per litre may be added if required.

The contractions of the ventricle are measured isotonically with a load of about 200 mg. The tension is not highly critical, a load sufficient to eliminate tonal effects at the temperature used being adequate.

Standard acetylcholine stock solution is made up as described by Wait, 1 mg. acetylcholine as the chloride or bromide per ml. of 5 p.c. sodium dihydrogen-phosphate is sealed in glass ampoules and put in a boiling-water bath for five minutes to sterilize it. This solution has a pH of 4, at which acetylcholine is stable, and keeps well in the refrigerator, being diluted in sea-water or distilled-water just before use. Such a solution has been shown not to vary significantly from freshly-made solution for at least eight months.

The solutions are added to the bottom of the bath by means of a syringe with a long needle. No more than 1 ml. should be added at any one time.

RESULTS.

Structure.

The muscle of *Tapes* ventricle is thin and arranged in a radiating pattern with two main centres of radiation. This feature is only seen in distended ventricles.

Histologically, the cells resemble those of vertebrate smooth muscle rather than cardiac, but their arrangement is rather like that of primitive cardiac muscle. Absolute identification with either type is, of course, not to be expected. Prosser described the ventricular muscle of *Venus* as being functionally and histologically most like primitive smooth muscle, and this description could probably equally well be applied to that of *Tapes*.

Effect of acetylcholine.

Small doses of acetylcholine were found to exert negative inotropic, chronotropic and tonotropic effects on the spontaneously beating *Tapes* ventricle, the most marked and regular effect being reduction of the beat amplitude. The effect on tone was noticed largely at lower temperatures when the tone is greater. Rate was decreased in a rather unpredictable manner, being apparently unaffected in many cases.

The effect on amplitude was the only constant feature ranging from no effect to complete cessation of beat within a small dose range (tenfold).

Sensitivity of the ventricle to acetylcholine varies slightly from animal to animal. No significant seasonal change in sensitivity was recognizable, but a slight increase seemed to occur from keeping the animals in the laboratory for more than a week. There was also an increase in sensitivity after a few hours' use.

The average ventricle showed complete cessation of activity with 10^{-9} acetylcholine, while extreme cases extended the range between 10^{-11} and 10^{-7}. After being set up for some four hours, the sensitivity rose gradually, thousandfold increases, i.e., from 10^{-9} to 10^{-12}, being common.

Effect of temperature.

Wait (1943) reported a temperature effect on sensitivity, claiming it as the reason for the seasonal variation seen by Prosser (1940). In *Tapes* no great seasonal variation in sensitivity was observed, but the effects of temperature were examined for comparison with *Venus*.

The range of temperatures studied was from 0° C. to 30° C. In general, the tone of the ventricle was higher at low temperatures, showing almost complete contraction at 0–2° C. and decreasing more or less logarithmically as the temperature rose. The temperature at which the base-line was even, tone being at a minimum, depended on the tension being applied to the ventricle. With the tensions normally used, tone was minimal at about 14° C.

Ventricular rate and amplitude showed an increase with rise in temperature. There is normally no detectable beat below 11–11·5° C. and near this level amplitude and rate are low. The rate is approximately doubled by a rise from 15–20° C., but amplitude changes little between 14·5 and 30° C.

Sensitivity to acetylcholine is changed very little between 0 and 30° C., although a fourfold increase was recorded in one experiment. Such alteration as was present agreed with Wait's findings, sensitivity being greater at lower temperatures.

Effect of other drugs.

Since a small amount of sodium dihydrogen phosphate is added with each dose of standard acetylcholine, it was considered advisable to test the effect of this drug on the ventricle. An amount of sodium dihydrogen phosphate equivalent to that added with acetylcholine to give a concentration of 10^{-5} was required to show any effect, smaller doses producing no discernible change. It was, therefore, considered that with the usual doses of acetylcholine used, the sodium dihydrogen phosphate effect could be disregarded.

Acetylcholine in dilute solutions is not very stable, breaking down to give acetate and choline; however, the solutions used appeared to last quite well, in some cases for over six hours. This unexpected stability led to an investigation of the effects of choline and acetate on the isolated ventricle. Acetate had no

demonstrable action even in relatively massive doses (0·5 p.c.). Choline, on the other hand, had an inhibitory effect, causing the ventricle to stop with concentrations of 10^{-4}; 10^{-5} choline showed slight reduction of the amplitude. It is noteworthy that following choline administration the rate was slowed markedly, much more so than after acetylcholine. Furthermore, recovery is very slow, the ventricle being hypodynamic for some hours afterwards. This response is seen to a very slight extent even with 10^{-13} choline.

Carbamylcholine chloride (Carbachol) also has an inhibitory effect on *Tapes* ventricle, the beat being slightly reduced by 10^{-8} and stopped by 10^{-7}, this being at a stage when the ventricle responded to 10^{-9} acetylcholine. Like choline, carbamylcholine showed a rather greater effect on rate than an equivalent dose of acetylcholine but, unlike choline, it was readily washed out.

It will be noted that the different activities of acetylcholine, carbamylcholine and choline are of the same order as those reported by Welsh and Taub (1948) for *Venus* heart.

As reported earlier for *Venus mercenaria* (Prosser, 1940), eserine has little effect on the sensitivity to acetylcholine, raising the level only by a factor of two or less which is insignificant with sensitivities of 10^{-9} or better. Besides this, the recovery of the ventricle from acetylcholine is inordinately slowed and fewer doses can be tested in a given time.

Also, as in *V. mercenaria*, atropine did not block the acetylcholine effect, indicating that the mode of action of acetylcholine is probably the same on *Tapes* as on *Venus*, the nicotinic actions predominating.

Adrenaline has marked positive inotropic and chronotropic effects and if the tension is such as to allow it, tone is also increased. This action can be used as an aid to starting refractory hearts when first set up. Unfortunately, adrenaline is easily washed out and the heart may not continue beating after washing. As Prosser (1940) pointed out, the ergot alkaloids also have a positive effect on *Venus* ventricle and show long persistence, surviving numerous changes of the medium. The ergot alkaloids also proved effective on *Tapes*, and a number of commercial preparations, mainly with combinations of dihydro-derivatives, were used with the same result.

Investigation showed no detectable change in sensitivity to acetylcholine immediately following ergotisation, but if excessive amounts of the alkaloids were added repeatedly the sensitivity was reduced.

As mentioned earlier, the medium used was sea-water, with or without added glucose. The sugar is only needed if the ventricle is to be used for long periods as it will beat for some hours without added glucose. A more readily available source of energy is alcohol which has a marked stimulating action on the ventricle, increasing amplitude several times in concentrations of 0·8 p.c. in the medium. This stimulation persists only as long as the alcohol is actually present. While the presence of alcohol shows no adverse effect on the acetyl-

choline-sensitivity of the preparation, it is not necessarily advised that the addition of alcohol to the sea-water be adopted as general practice. Of the alcohols tested, ethyl alcohol had the greatest effect, propylene glycol and isopropyl alcohol being only slightly less active.

DISCUSSION.

From the results presented it may be seen that the physiology and pharmacology of the ventricle of *Tapes* are very similar to those reported for *Venus*. It therefore seems reasonable to use *Tapes* as a local substitute for *Venus*, being guided by the data already published on *Venus* both for application of method and pharmacology.

As an assay method for acetylcholine, the use of *T. turgida* is closely comparable to *V. mercenaria*. The range of sensitivity is almost the same (Smith and Levin, 1938; Wait, 1943; Prosser, 1940); it is reliable, rapid, easily set up and, as far as can be said at this stage, seems to have the same specificity.

The increase in sensitivity over several hours has not been reported for *V. mercenaria*, but is a constant feature for those specimens of *T. turgida* tested in this laboratory. This would necessitate alternating tests of standard and unknown, but as this is already the usual practice in assays of this type, it cannot be considered a great disadvantage. Moreover, since the drug need only be applied for one minute and the ventricle rested five minutes between tests (Wait, 1943), a test may be completed in 30–40 minutes, during which time no perceptible change in sensitivity can occur.

Like *V. mercenaria*, *T. turgida* offers a simple test of high sensitivity, not comparable with that of frog lung (Corston, 1940), but much more reliable. It also compares favourably with the other assay methods generally available.

The temperature effect reported by Wait (1943) for *V. mercenaria* could not be satisfactorily shown for *T. turgida* and, if his explanation of the phenomenon is correct, this may indicate an even lower choline esterase level than that in *V. mercenaria*, itself very low in choline esterase (Smith and Glick, 1939). This suggestion is supported by our finding of less effect from eserine. In *T. turgida* we found eserine had very slight effect, increasing sensitivity two-fold at most, and usually less, while Prosser (1940) indicated a rise of "several times." In both animals the major effect seems to be slowing of recovery rate.

Apart from its use in bioassay, *V. mercenaria* has been used in more fundamental research as a test object for acetylcholine substitutes and to elucidate the mode of action of acetylcholine (Welsh and Taub, 1948, 1950, 1951, 1953; Welsh and Slocombe, 1952). *T. turgida* is equally well suited to such investigations and may be regarded as a new tool for such lines of research in the regions in which it is available.

The discovery of such a satisfactory substitute for *V. mercenaria* indicates the possibility of finding other test animals in regions in which neither mollusc

is found. Though in this case we obtained our greatest success with a Venerid, there is no reason why this should be the only family tested. Prosser (1942) states that the hearts of Mollusca in general are of the same type (myogenic but innervated) and are inhibited by acetylcholine. Success was reported in a very different family of molluscs by von Euler, Chaves and Teodosio (1952), who reported a response to 10^{-9} acetylcholine by the heart of a sea-hare *Aplysia dactylomela,* which is in a different zoological order to the Veneridae.

SUMMARY.

Tapes turgida is suggested as a substitute for the closely related mollusc *Venus mercenaria* as a test object for acetylcholine.

The structural, physiological and pharmacological characteristics of *Tapes turgida* are compared with those of *V. mercenaria* and found to be very similar.

Sensitivity to acetylcholine is shown to be approximately the same for both molluscs and the specificity, as far as it could be tested, seems comparable.

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OBSERVATIONS ON THE CRITICAL TEMPERATURE FOR VASOMOTOR REACTION IN THE FINGERS OF CHILBLAIN SUBJECTS

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The observation that the hands of some persons subject to attacks of chilblains in the winter were frequently much cooler than those of normal persons, even at other seasons, prompted an investigation into the digital circulation of a group of such people. Comparisons were made between the responses of normal and chilblain subjects to different environmental temperature, to local hand cooling, and to exercise; and the response of the digital circulation was assessed from measurements of skin temperature.

The circulation of the hands and feet has been found to play a part in regulating heat loss and maintaining a constant body temperature over a certain range of environmental temperature, and under standard conditions this range has been found to be fairly constant for any individual (Sheard, Williams and Horton, 1941). The lower limit of this air temperature range, referred to as the *critical temperature*, was measured in subjects as close as possible to their normal everyday conditions of living. The response to cooling the hand in water at 18–20° C. was measured under conditions found by Spealman (1944) to result in maximum vasoconstriction without hyperaemic response (Lewis, 1936). From the subsequent behaviour the relative significance of general and local vasoconstrictor influences was inferred. The relation between the digital circulation and the increased heat dissipation following exercise was also examined.

Methods.

Subjects were 30 medical students, 24 male and 6 female. One half were normal, and one half had been subject to chilblains.

Experiments were carried out in a small soundproof room where temperature could be controlled to within ±0·5° C. Air movement was from a shielded fan, and seldom exceeded 5–10 metres per minute over any part of the body.

Skin temperatures were measured by nichrome-constantan thermocouples, constructed of 40 S.W.G. wire for their final centimetre, with a tiny globule of silver solder at the actual junction. They were fixed to the nail beds of the fingers, to the forehead, and to the chest by a thin film of collodion.

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For all experiments the subjects were dressed as closely as possible to the normal male clothing of cotton underclothes, long-sleeved shirt, and long trousers, shoes and socks. The coat was removed. Subjects were warned not to undertake any strenuous activity in the hour before an experiment, and any sickness (e.g., even a mild cold) was considered sufficient to exclude a subject until recovery was complete.

In measuring the critical temperature, the room temperature was at first maintained at about 2–3° C. above the expected temperature. If the hands were cold on arrival, their warming was hastened by immersion in a bowl of water at 37° C. for 2 minutes so that delay was minimized. The subject then lay on a fibre mattress with his hands on copper gauze supports and the thermocouples were affixed. About 10–15 minutes were then allowed for him to recover and for the fingers to attain a steady temperature, and the room was then cooled at 1° C. per 20 minutes. Finger temperature fell gradually, with oscillations in many subjects, but finally there was a rapid fall (comparable to the rate obtained with a sphygmo-manometer cuff at 180 mm. round the upper arm). The air temperature at which this final cooling commenced was taken as the critical temperature, and experiments showed that if the room was held at this temperature the fingers remained about 5° C. higher, whereas they approximated the air temperature at levels about 1° C. below the critical temperature. The experiment was usually completed within about 60 minutes of the subject's arrival.

In the experiments on local cooling, the initial preparation was the same as above. When a steady finger temperature was attained, the right hand was immersed for a total of 10 minutes in water at 18–20° C., which was gently stirred for a few seconds half-way through the immersion period. At the end of the immersion the hand was removed, wiped dry, and its temperature observed for the next 15–20 minutes. Room temperature was maintained constant throughout any single experiment, and several experiments performed on each subject at different room temperatures.

Exercise was performed on a bicycle ergometer, at a basic rate of 600–900 kgM/minute for a 70 kg subject, with proportionate adjustment to the individual weight. The duration of exercise was 5′. Several experiments were performed with each subject, each at a different room temperature below the critical temperature, to determine the lowest room temperature at which the fingers would warm up by 5° C. within the 15 minutes following the exercise.

Metabolism was determined with a Sanborn Metabolimeter.

Statistical analysis was performed according to the methods of Fisher (1946).

RESULTS.

The critical temperature.

The reproducibility of the critical temperature estimation was studied with three subjects, for whom the means and standard deviations were 21·4° C. ±0·7 (Std. Deviation), 25·5° C. ±1·1, and 24·0° C. ±0·4, for 6–8 experiments each. During a period of mild chronic upper-respiratory-tract infection, with hay fever and some loss of sleep, 4 experiments on the first subject gave a mean critical temperature of 22·9° C. ±0·6, which is significantly higher than his normal ($P<\cdot001$).

Two determinations of the critical temperature were then performed for each member of the entire group, about one month apart, each experiment taking about 45–60 minutes. A further experiment was carried out with all but 3

subjects in which the total duration was increased to about 150 minutes, to determine the effect of more prolonged rest. The results of these experiments are shown in Table 1.

For the short experiments the mean and standard deviation for the normal group are 21·9° C. ±1·6, and for the chilblain group 24·2° C. ±1·2, a highly significant difference ($P<·001$). There was no systematic difference between the June and July findings, the differences being randomly distributed around

TABLE 1.

Critical Temperatures in Long and Short Experiments, in °C.

Subject No.	June	July	Difference	Long Expt.	Duration (Minutes)
Normal Subjects					
1	20·3	22·8	−2·5	—	—
2	22·4	22·8	−0·4	24·3	160
3	18·8	18·2	+0·6	22·2	150
4	—	20·7	—	22·8	160
5	19·9	21·5	−1·6	24·2	140
6	20·9	—	—	24·3	140
7	—	24·0	—	24·9	160
8	22·7	23·3	−0·3	21·2	130
9	21·0	21·3	−0·3	21·2	130
10	23·8	24·6	−0·8	24·2	140
11	22·2	24·1	−1·9	24·3	150
12	23·6	21·5	+2·1	—	—
13	20·2	20·2	0·0	21·3	210
14	22·2	20·6	+1·6	22·4	150
15	24·3	23·8	+0·5	25·8	180
Mean	21·9		−0·2	23·3	160
Std. Dev.	1·6		1·4	1·4	
Chilblain Subjects					
1	22·8	23·3	−0·5	24·8	180
2	23·2	23·8	−0·6	23·3	180
3	25·5	24·8	+0·7	26·3	160
4	22·7	21·8	+0·9	24·3	100
5	25·3	—	—	26·4	140
6	25·0	25·5	−0·5	23·3	170
7	22·8	22·3	+0·5	24·2	120
8	25·3	24·5	+0·8	25·4	160
9	22·6	24·5	−1·9	24·3	130
10	24·0	25·6	−1·6	26·5	120
11	22·3	22·6	−0·3	25·2	120
12	25·3	—	—	25·8	110
13	23·7	—	—	23·0	180
14	25·8	—	—	—	—
15	26·5	24·5	+2·0	25·5	150
Mean	24·2		0·0	24·9	140
Std. Dev.	1·2		1·1	1·2	

zero, with variance somewhat greater than was found for the three individual subjects studied in greater detail. For the long experiments there was an increase in the critical temperature in the normal subjects amounting to 1·4° C. ($P=\cdot01$), and in the chilblain subjects to 0·8° C. ($P<\cdot05$). The difference between the two groups was reduced to 1·6° C. ($P<\cdot01$).

The response to exercise in the two groups was not significantly different; the minimum air temperature for post-exercise warming of the hands was 16° C. ±3 for the normal group and 17° C. ±2 for the chilblain group. Variations in build, training and fitness, etc., probably account for the great intra-group variations. There was a difference noted in the behaviour of the ear circulation, in experiments where there was post-exercise finger warming. In none of 16 experiments of this type with the normal subjects did the ear temperature rise above 25° C., whereas in 14 out of 25 such experiments in the chilblain group the ears warmed from similar pre-exercise levels to above 30° C., for which behaviour $P<\cdot001$.

Over a range of air temperature from 18–29° C., the relationship between the forehead (F) and air temperature (A) may be expressed by the regression equations

$$F=34\cdot8+0\cdot19\ (A-23\cdot6) \quad \ldots\ldots \quad \ldots\ldots \quad (1)$$

and $$F=35\cdot1+0\cdot18\ (A-25\cdot2) \quad \ldots\ldots \quad \ldots\ldots \quad (2)$$

for the normal and chilblain subjects respectively. These regression equations are each highly significant ($P<\cdot001$), but there is no significant difference either in their slope ($P=\cdot5$) or in the actual forehead temperature predicted for any given air temperature. Similar regression equations were obtained for the chest temperature, but the actual skin temperatures were of the order of 0·5–1° C. higher in all cases.

Using a Sanborn metabolimeter, no differences were found between the two groups in either basal metabolism, or metabolism under the conditions of the critical temperature determinations (normals, 159, chilblain group 154 ml. O_2/square meter/minute, $P=\cdot15$). Only in the normal subjects was there a relationship between critical temperature (C) and oxygen consumption in ml./square metre/minute (M),

$$C=21\cdot9+\cdot09\ (M-159) \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (3);$$

for this regression $P=\cdot02$.

The recovery temperature.

When one hand is dipped in cold water and then removed after 10 minutes, three different responses are possible during the ensuing 15 minutes. The unimmersed hand may remain warm and the immersed hand warm up after removal (Fig. 1), the unimmersed hand may begin to cool during the immersion and after removal both hands are cold and remain so (Fig. 2), or the unimmersed hand may remain warm and the immersed hand cold (Fig. 3). A series of

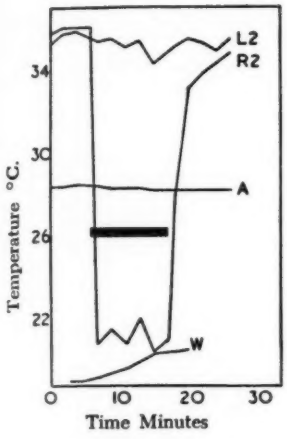

Fig. 1. The effect of immersion (shown by bar) of the right hand in cold water. Subsequent warming of right index finger (R2) almost to level of left (L2). Air temperature (A) and water temperature (W.).

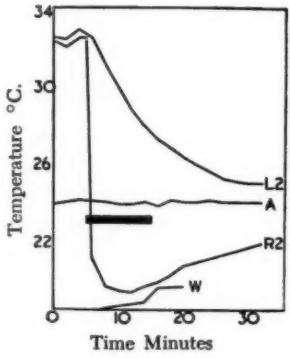

Fig. 2. The effect of immersion (shown by bar) of the right hand in water. Showing cooling of both hands.

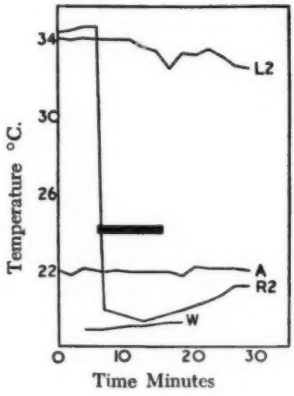

Fig. 3. The effect of immersion (shown by bar) of the right hand in water. Right hand remains cold, left hand remains warm, after removal from water.

experiments was carried out with each subject, each one at a different room temperature, to ascertain the lowest air temperature at which the immersed hand would still show significant warming above air temperature during the 10–15 minutes immediately after removal from the water. This is defined as the *recovery temperature*, and it was estimated to the nearest 1° C., because the day-to-day variation in the subject's responses (e.g., in the critical temperature determination) was of this order. Table 2 shows the results in both the chilblain and normal groups; only 26 subjects were available for these experiments.

There is a mean difference of 3·4° C. between the recovery temperature of the two groups of subjects ($P<\cdot001$). This is a larger difference than that between the critical temperatures, mainly because the recovery temperature is higher than the critical temperature in the chilblain group, but much the same in the normal group. Included, therefore, in Table 2 are the differences for each individual subject between these two temperatures (R–C), and it may be seen that the mean value of this difference is not significant for the normal subjects ($P=\cdot2$), but for the chilblain subjects it is significant ($P<\cdot01$). The meaning of this difference will be discussed later.

It is to be emphasized that this difference in the behaviour of the two groups of subjects was manifest only under a restricted range of conditions; if the

TABLE 2.

Recovery (R) and Mean Critical (C) Temperature for Normal and Chilblain Subjects, ° C.

Subject No.	Normal Subjects			Chilblain Subjects		
	R	C	R-C	R	C	R-C
1	21	20·3	+0·7	26	23·1	+2·9
2	22·5	22·6	−0·1	24	23·5	+0·5
3	21	18·5	+2·5	26	25·3	+0·7
4	22	20·7	+1·3	24	22·3	+1·7
5	20	19·9	+0·1	27	25·3	+1·7
6	20·5	20·9	−0·4	25	25·3	−0·3
7	24	24·0	0·0	23	22·6	+0·4
8	22	23·0	−1·0	25	24·9	+0·1
9	21	21·2	−0·2	25	22·6	+2·4
10	24·5	24·2	+0·3	25	24·8	+0·2
11	24	23·2	+0·8	26	25·3	+0·7
12	23	22·5	+0·5	26	23·7	+2·3
13	20	20·2	−0·2	26	25·8	+0·2
Mean	21·9	21·6	+0·33	25·3	24·2	+1·04
Std. Dev.	1·6	1·6	0·88	1·1	1·2	1·03

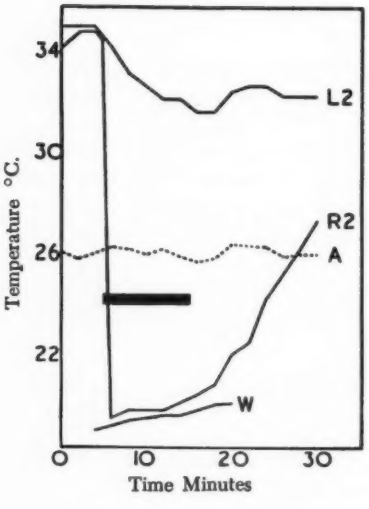

Fig. 4. The effect of immersion (shown by bar) of the right hand in water. Slow, even warming after removal.

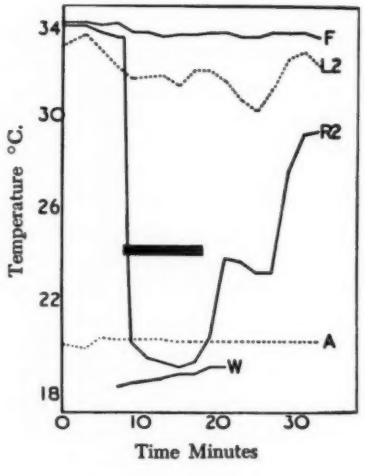

Fig. 5. The effect of immersion (shown by bar) of the right hand in water. Warming occurs in steps.

water was too cold or too vigorously stirred, very thorough chilling of the hand resulted, so that even in the normal subjects recovery was delayed longer than the 15 minutes available for observation after removal from the water. Accordingly, stirring was restricted to a single 5-second bubbling by compressed air at half-time.

A further difference in behaviour was observed in those subjects with whom an experiment was performed close to the actual recovery temperature. This occurred in 9 of the normal subjects and 10 of the chilblain subjects. Warming of the immersed hand under these circumstances occurred either gradually, as shown in Fig. 4, or in a series of steps, as shown in Fig. 5. The unimmersed hand usually preserved a fairly even temperature in the "gradual rise" type of experiment (Fig. 4), but in the "step rise" type of experiment (Fig. 5) the unimmersed hand usually cooled during the horizontal part of the step, and warmed again during the vertical part. The step rise occurred in 6 of the 9 normal subjects, but only 1 of the 10 chilblain subjects, on whom an experiment was performed at the actual recovery temperature, the gradual rise occurring in the remainder. For this distribution, after applying the correction for continuity, we obtain $\chi^2=4\cdot33$, $P<\cdot05$, which means that there is a significant difference between the two groups in their type of behaviour at the recovery temperature.

DISCUSSION.

It is difficult to compare the critical temperature as measured in this series of experiments with figures from overseas authors because of widely differing conditions of clothing and the frequent employment of the basal metabolic state. Many American workers (Roth, Williams and Sheard, 1938; Sheard and Williams, 1940; Sheard, Williams and Horton, 1938, 1939, 1941; Freeman and Nickerson, 1938; Ferris, Forster, Pillion and Christenson, 1947; Winslow, Herrington and Gagge, 1938) have found that the fingers of the lightly-clothed basal subject begin to exceed air temperature when the latter rises above about 20–23° C., whereas in the present group the critical temperature averaged 22° C. under normal conditions of dress, diet and activity, and rose to almost 24° C. after 2 to 3 hours rest, when the basal rate was still not attained (Gubner, di Palma and Moore, 1947).

Again, English figures (Lewis, 1936, 1941; Pickering, 1933) for normally clothed and active subjects are of the order of 16–18° C., as against the mean here of 22° C. It is suggested that this difference may be related in some way to the less extreme nature of the climate in Sydney, where the outdoor winter temperature during the day is seldom much below 10° C. Burton (1940) has described a slow acclimatization to high environmental temperature, manifested by a fall in the mean skin temperature at any given air temperature.

The difference between the normal and the chilblain subjects appears to be confined to the fingers under the conditions of the experiments; the responses of the toes were too sluggish to be investigated by the present technique, and the trunk (at least the forehead and chest) behaved similarly in the two groups. From the relationship between critical temperature and metabolism (equation (3)), a difference of metabolism of some 15–20 p.c. would be expected for the difference in critical temperature found between the two groups. Instead, we find a statistically insignificant difference of only 3 p.c., which suggests that we are dealing with a group in which there is some fundamental difference in the mechanism responsible for the critical temperature phenomenon. It is possible, from the results of the long-term experiments, that this difference may disappear under basal conditions, and that it may represent a different response to the "active" state. Careful and repeated questioning elicited no differences in the activity of the two groups of subjects. Attempts to determine critical temperatures during sleep (when such influences would be relaxed) were unsuccessful as few subjects could sleep at short notice during the day; there did appear to be some lowering of the critical temperature for 3 chilblain subjects who were able to do so.

There is also evidence, derived from the water immersion experiments, for local hyper-reactivity to cold on the part of the skin vessels in the chilblain subjects. When the immersed hand is removed from the water, it is subject to vasoconstrictor effects from two sources; the general vasomotor tone, which may be expected to act equally on both hands, and the local effect of the cooling, which should act on the immersed hand only. We can explain the behaviour seen in Fig. 3 on this basis—the general vasomotor tone is sufficient to restrict the finger circulation, so that the left finger temperature is only 33° C., and the additional effect of local cooling results in keeping the right hand cool for at least 15 minutes after removal from the water. This, then, is a record of an experiment carried out between the critical temperature and the recovery temperature. Had the room been a little warmer, the immersed hand would have warmed; had it been a little cooler, the unimmersed hand would not have remained warm.

In 6 experiments on chilblain subjects and 3 on normals in which the room temperature was between the critical temperature and recovery temperature, responses of the type shown in Fig. 3 suggested a local vasoconstrictive effect of cooling on the digital vessels for both groups. The difference between the room temperature at which the right hand first shows diminution of vasoconstriction after standardized local cooling (recovery temperature, "R") and the mean temperature at which the finger vessels are almost completely restricted without previous cooling (critical temperature, "C") should give a measure of the strength of the local constrictor effect of such cooling. Table 2 shows that there was no significant difference (R–C) in the normals, but a mean difference of

1° C. ($P<\cdot01$) in the chilblain group. This test, while not sensitive enough to reveal the local effect of cooling in normals (shown in selected experiments described above) showed that there was a significant difference between normals and chilblain subjects. The difference in the type of warming at the recovery temperature also suggests a local hypersensitivity to cold in the chilblain group, whose finger temperature rose very gradually at first; in normals there was a step-wise rise of temperature interpreted as due to intermittent release of vasoconstriction, under the influence of the vasomotor centre.

To summarize, then, the hands of chilblain subjects are cold at air temperatures on the average 2–3° C. above normal controls, and their blood vessels are also more responsive to local cooling, so that, once cold, they are harder to warm up again. This means that they may fail to warm up at air temperatures where, once warmed, they would remain so.

Whilst it is not claimed that this factor is the only one operative in the causation of chilblains, it is suggested that it is of considerable significance. It has been found during war-time that the lesions of trench and immersion foot are similar in their pathology to the chilblain, but greater in severity, and they occur in almost all persons exposed to sufficiently cold conditions for a sufficiently long time (Ungley, 1943). It is seen that chilblain sufferers, on the whole, will have colder hands than normals for a considerable part of the winter, and will therefore be more likely to suffer tissue damage as a result.

SUMMARY.

The lowest air temperature at which the temperature of the fingers exceeds that of the air by 5° C., referred to as the critical temperature, has been determined in a group of normal subjects and a group subject to chilblains.

The mean critical temperature for the normal subjects is about 22° C. under normal conditions of dress, activity and diet, a higher figure than is reported in overseas literature. It is suggested that this may be related to climate.

The chilblain subjects had an average critical temperature in excess of 24° C., with no difference detected in the behaviour of the trunk temperature or body metabolism. There appears, therefore, to be a difference in the vasomotor control of the hand circulation.

Evidence is also presented that there is a direct vasoconstrictor effect persisting after local cooling, which is more powerful in chilblain subjects than in normals.

The significance of this behaviour in the genesis of chilblains is discussed.

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CRANIAL WINDOWS IN THE RAT

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In the course of a study of the cerebral complications of hypertension (Byrom, 1954), the need arose to obtain serial photographs of the cerebral arteries over periods of weeks or months. To meet this need the cranial window technique, used in larger animals by physiologists for over a century (Ravina, 1811), has been extended to the rat.

Three methods will be described. In the first the inner table of the skull is used as a window. In the second a window is made of flat sheet perspex and inserted into an accurately cut hole in the skull. In the third the window is moulded in transparent acrylic resin to restore the normal internal contour of the individual skull.

GENERAL PROCEDURES.

Preparation of the rat. Anaesthesia is induced with ether and maintained with ether-oxygen mixture from a small warmed vaporizer, except in the case of moulded windows, when

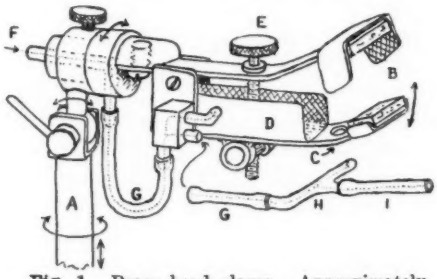

Fig. 1. Brass head clamp. Approximately full scale. (A) Spindle to fit bush at head of rat board; (B) cork-lined jaws; (C) hole for upper incisor teeth; (D) side walls of clamp, made of shim brass; (E) locking screw; (F) nipple for ether-oxygen mixture, the path of which is indicated by the continuous dotted line; (G) polythene tubing; (H) glass T-tube and (I) soft rubber tube to pharynx.

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subcutaneous nembutal (6 mg.), repeated if necessary, is supplemented with ether and oxygen. Respiratory obstruction by mucus is relieved when necessary by suction through a glass cannula.

Operating table. The anaesthetized rat is held in the prone position by means of a clamp (Fig. 1) which grips the maxilla firmly between rubber or cork-lined jaws. The clamp is mounted on a universal joint at one end of a wooden or perspex rat board (12×8×¾ inches), the head of the rat being over the centre of the board. The belly is supported by a suitable sling. The rat board is mounted on a central vertical spindle which can be rotated in a bush in the main operating table. Saline at 38° C. is circulated from a large Dewar flask, containing a pump, heating element, and thermostat, through wide (6 mm. bore) polythene tubing to an irrigating head mounted on an inverted U-shaped magnet which can be moved about on a sheet of galvanized iron screwed to the surface of the rat board. From the irrigating head a short length of polythene tube (0·5 mm. bore) drips saline on to the operation site when required. A rectangular depression in the centre of the board is drained through the central spindle to a waste bottle suspended under the table. Oxygen-ether mixture is delivered to the space between the jaws of the nose-clamp and also along the cross limb of a small glass T-tube to the remaining limb of which is attached a short piece of soft rubber tube (4 mm. bore) which is inserted in the pharynx. The tubes conveying saline and anaesthetic are brought up the central spindle to the space between the table and the board and thence over the edges of the board to the upper surface. A binocular Greenough microscope with overall magnifications of 7 and 15 diameters, mounted on an extension arm, can be swung over the field when needed. The field is illuminated by a 70 watt excitor lamp with an adjustable condensing lens mounted on a retort stand clear of the rat board.

A dental drilling machine is also mounted on the main table. This should be of the cord and pulley variety, with full range of movement and equipped with a contra-angle handpiece and a miniature head. The speed of the motor is controlled by a foot-operated rheostat.

Exposure of the skull. The skin over the dorsal surface of the skull is shaved, a median incision is made and the skin and subcutis are reflected by blunt dissection and kept apart by small weighted retractors. The pericranium is scraped off the skull.

EXPERIMENTAL.

Method 1. "Bone" windows.

The simplest way of observing the surface of the brain is to remove the outer layers of the dorsal surface of one parietal bone until only a thin shell of bone remains. The dura, though tough, is almost transparent and a remarkably clear view of the cerebral vessels is obtained (Fig. 13). The most suitable tool is a fine-toothed dental "wheel" burr (Fig. 2a), the corners of which have been slightly rounded by rotating the burr against a hard fine grindstone.

The operation is performed under the higher power of the microscope, with the saline drip running freely. Using a circular movement, the side of the burr is made to generate a saucer-shaped depression in the centre of one parietal bone, which is gradually deepened and widened until the surface of the brain is clearly visible. The exposed bone does not bleed. Impending penetration of the inner table can be detected by cautious pressure with a fine probe.

The main advantages of the bone window are the extreme simplicity of the technique and the fact that the skull is neither opened nor deformed. It is subject to several limitations.

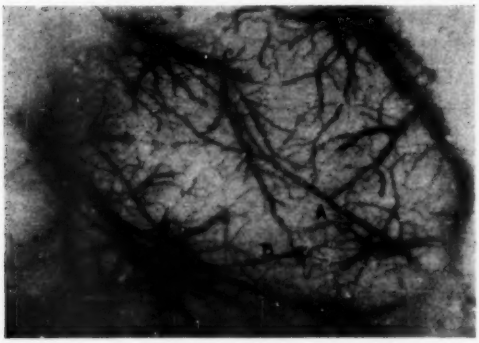

Fig. 13. "Bone" window showing (A) branches of middle cerebral artery, (B) meningeal (dural) vessels, (C) cerebral veins. ×15.

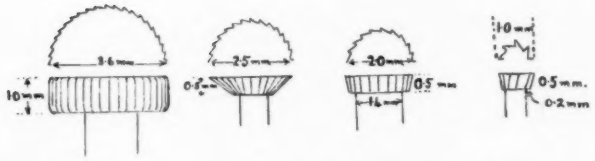

Fig. 2. Steel burrs. (a) Fine-toothed dental wheel burr used in making "bone" windows. (b) and (c) Specially made burrs for cutting bone flap for acrylic windows. (d) May be used instead of (c): it is a standard inverted cone burr ground down to the dimensions shown. All burrs must fit the contra-angle handpiece.

First, the window does not remain clear indefinitely. For several days the cavity contains only organizing blood clot and granulation tissue, which is easily removed. This is later replaced by dense and very vascular connective tissue which bleeds freely when detached. Eventually new bone formation occurs in the connective tissue, especially if the inner table has been breached, and also in the form of leaf-shaped ingrowths from the outer table round the edge of the window. However, the newly-formed tissue often remains separated from the surface of the window by a distinct plane of cleavage and it is often possible, even after several weeks, to expose most of the window by scraping without recourse to the burr. Secondly, the vascular pattern is somewhat confused by the presence of meningeal (dural) vessels. Thirdly, the vibration of the burr may cause temporary arterial contraction, especially in hypertensive animals. In studies concerning the calibre of the vessels the window should therefore be made several hours before the experiment. The skin incision must of course be closed between observations.

Method II.—Flat perspex windows.

These consist of rectangular plates of thin sheet perspex inserted into accurately cut holes in the bone after reflecting the dura. Two kinds have been used.

Flanged windows are made as follows: The thinnest sheet perspex available commercially has a nominal thickness of 1 mm. From the thinnest part of a sheet a piece about two inches square is clamped to a rectangular block of brass, which is mounted in the milling attachment of a lathe, preferably a jeweller's or micro-lathe. A dental fissure burr, 2 mm. in diameter, is mounted in a collet in the headstock and with this vertical and horizontal grooves 0·6 mm. deep are milled in the perspex sheet. These grooves are spaced to leave rectangular projecting areas measuring approximately 5·0×3·5 mm. To avoid overheating, each groove is milled in several stages. In this way a sheet of 12 to 16 windows is made. When a window is needed it is cut from the sheet with a fine jeweller's saw. The edges and corners are rounded off with a fine jeweller's pivot file and shallow grooves for the retaining wires are cut with the saw (Fig. 3). The window is washed in hot soapy water to remove the protective film of gelatine, rinsed, dried, and wrapped in cellophane until needed.

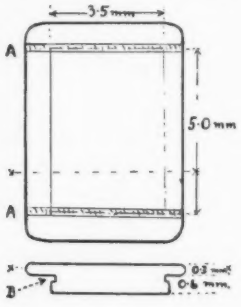

Fig. 3. Flanged type of flat perspex window. (A) Grooves for retaining wires. The window may be undercut as shown (B).

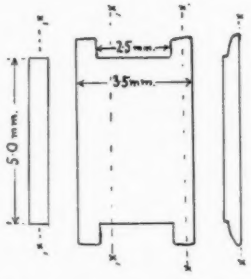

Fig. 4. Flush-type of flat perspex window.

Flanged windows are quite satisfactory for comparatively short experiments, but the upper surface of the window and the retaining wires project slightly above the surface of the skull and after about 4 to 6 weeks the window tends to work loose.

Flush windows (Fig. 4) were developed to overcome this drawback. These are thinner (0·5 mm.) so that the upper surface is flush with the surrounding bone and the flange is replaced by four small lugs which rest in grooves cut in the bone. Flush windows are more troublesome to machine because the com-

mercial sheet perspex must be milled to the correct thickness and polished before the grooves are cut. If facilities are available they may be cast in transparent acrylic resin in suitable moulds.

Inserting flat windows. To ensure that the aperture to be cut in the skull shall conform accurately with the dimensions of the window, two small locking screw calipers (Fig. 5) have been made. These are set to the exact length and width of the window as measured with a micrometer.

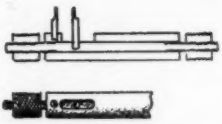

Fig. 5. Locking screw caliper, made of steel, approximately full scale.

Fig. 6. Cutting the bone flap. (a) The area is marked out; (b) the groove is deepened to approach the inner table; (c) the inner table is cut through.

The bone flap may be cut with dental "wheels" and "inverted cone" burrs of suitable sizes. Standard burrs may be used, but finishing burrs, which have finer teeth, are preferable. The burrs are ground to shape as shown in Fig. 2b, c, and d. We have found it more satisfactory, however, to make our own cutters from silver steel rod in the jeweller's lathe, using inverted cone burrs to cut the teeth, the finished cutter being case-hardened.

The flap is cut in three stages, as shown in Fig. 6, in the dorsal surface of the right parietal bone. The first step is to mark out a rectangular area on the dorsal surface of the right parietal bone conforming as closely as possible with the dimensions of the window as indicated by the two calipers. The bone is sponged with saline at frequent intervals and blotted dry between each cut. An assistant blows the bone dust away with a narrow glass tube mounted in a rubber hand bulb. The groove is now deepened to approach the inner table, the dimensions and angles being frequently checked and corrected when necessary, the objective being an aperture in which the window will be a tight press fit. If a "flush" window is to be used, the longitudinal grooves are extended slightly beyond the front and back edges of the aperture to accommodate the four lugs.

Finally the inner table is cut through along the outer borders of the grooves. This must be done very carefully under the higher power of the microscope to avoid tearing the dura. Penetration of the bone is usually indicated by the appearance of a little clear fluid at the bottom of the groove, but brisk haemorrhage at this stage does not necessarily mean that the dura has been perforated. The anterior and posterior grooves should be cut through first, then the lateral, and finally the median groove. In each case the cut should be commenced about the middle of the groove and extended backwards

towards the operator, the anterior end being left to the last. When the bone flap yields to cautious pressure at several points no further cutting is needed.

The retaining wires. Before removing the bone it is advisable to drill the holes for the retaining wires. For this purpose a jeweller's "spade" drill, size 2½ (0·25 mm.) is mounted in a special adaptor (Fig. 7) which fits the dental

Fig. 7. Adaptor for jeweller's spade drill. (A) Jeweller's spade drill, 0·25 mm. The shoulder of the drill should be ground back, as shown, and the shaft filed slightly flat opposite the grub-screw. (B) Grub-screw, 0·5 mm. metric thread, in head of adaptor. (C) Shaft of adaptor, machined to fit contra-angle handpiece. The adaptor is made of silver steel.

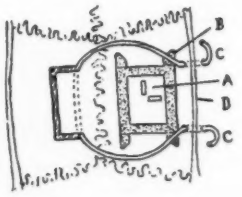

Fig. 8. Operation site before removing bone flap. (A) Bone flap showing central "key" grooves used in making moulded windows; (B) groove for lug; (C) retaining wire, showing method of fixation; (D) temporal crest.

handpiece. In using these fragile drills the risk of breakage is lessened if the site is irrigated with saline and if the drill, revolving at high speed, is withdrawn at frequent intervals.

The two lateral holes are drilled obliquely through the temporal crests exactly opposite the anterior and posterior edges of the aperture after detaching the fibres of the temporal muscle from the lateral aspect of the crest (Fig. 8).

On the medial side of the window the wires may be secured in one of three ways. The first and simplest way is through holes in the opposite temporal crest. This method is only satisfactory for brief experiments because the anchorage is too far from the median edge of the window and the wire works loose in time. In the second method a U-shaped groove about 0·25 mm. wide is cut with a dental "wheel" burr in the opposite parietal bone (Fig. 8). This method is satisfactory for flanged windows.

The third method is used with flush windows and also with the individually-moulded windows which will be described later in this paper. In this method the two median holes are drilled horizontally between the two tables of the *right* parietal bone. In order to do this two transverse grooves, semicircular in section, are made in the left parietal bone and sagittal suture (Fig. 9). The grooves for the retaining lugs are also broadened medially as shown. The holes

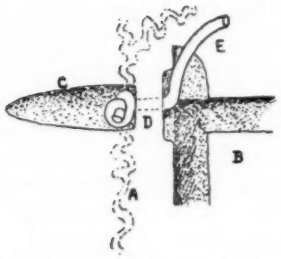

Fig. 9. Method of anchoring median end of retaining wires, as used with moulded windows. (A) Sagittal suture; (B) bone flap in right parietal bone, ready for removal; (C) groove in left parietal bone; (D) hole drilled horizontally between outer and inner tables of bone; (E) retaining wire knotted and threaded ready for tightening.

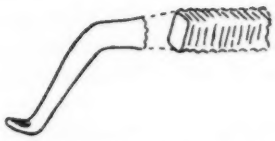

Fig. 10. Dental probe adapted for scraping cut edge of inner table of skull. The end of the probe is approximately 1 mm. long by up to 0·5 mm. thick, and its upper surface has been filed flat.

are then drilled. The wires, of pure annealed silver, or preferably gold, 0·20 mm. thick, are threaded through the holes and bent clear of the field. The bone flap is now gently raised with the blade of a tenotome, grasped with beaked forceps and removed.

Saline irrigation of the field is started. The cut edge of the inner table of the skull is scraped clear of spicules of bone and dura. A suitable scraper is shown in Fig. 10. Debris is clearly out of the cavity with a camel-hair brush. The window is next tested for fit and filed down if necessary. At this stage the operator may be tempted to wire the window into position, for the dura is virtually transparent. Whenever this has been done, however, it has been found later that the field has become obscured by oozing from small nutrient arteries torn in detaching the bone, and since no means of preventing this has been found, it is necessary to open and reflect the dura. This calls for very great care, for the exposed brain is very vulnerable and bleeds at the slightest injury.

Fig. 11. Probe-pointed cutter for extending incision in dura. Diameter approximately 0·35 mm.

Two instruments are necessary. The first is a fine sewing needle ground down to a narrow very sharp point on the jeweller's lathe under the dissecting microscope. The second is a similar needle which has been converted into a probe-pointed cutter (Fig. 11). Both instruments must be very highly polished and preserved from corrosion. They may be mounted in small brass handles. With the needle tangential to the surface a slit is pricked and torn in the dura about the centre of the field. The cutter is inserted through the slit and used to

extend the opening diagonally into each of the four angles of the aperture. If saline is now allowed to drip on to the surface of the exposed brain the four flaps will separate and can be drawn back gently to lie clear of the aperture. The medial flap should be retracted with special care because tributaries of the sagittal sinus sometimes cross the subarachnoid space to enter the dura at some distance from the sinus. The window may now be laid gently on the surface of the brain and pressed into position. The retaining wires are now drawn tight, bent over the temporal crest and cut short. The skin is sutured with fine cotton thread, the stitches being removed 7 days later.

Advantages and drawbacks. Flat perspex windows are the most satisfactory type for general purposes. With the right instruments and optical aid the technique is easily mastered and a window can be inserted in an hour or less. After the operation a dense membrane, continuous with and evidently derived from the dura, quickly grows over the *outer* surface of the window, and this must be incised and reflected at subsequent operations. The inner surface of the window usually causes no visible reaction, and the windows remain optically perfect even after many months and are thus suitable for chronic experiments. The main drawback of the flat window, apart from the tendency of the flanged variety to work loose, is that it does not accurately restore the contour of the vault of the skull. However, the dorsal surface of the parietal lobe is very nearly flat and distortion is usually minimal or absent provided that the volume of the brain remains normal. But if the brain swells, as in hypertensive encephalopathy, significant distortion of the cerebral vessels may occur.

Method III. Individually-moulded windows.

The preparation and insertion of a moulded window takes several hours and is done in two stages. In the interval the skin is sewn over a temporary flat window and the rat is kept lightly anaesthetized with nembutal.

After exposing the skull and stripping the periosteum a rectangular area is marked out as previously described. Accuracy is not necessary provided that the area is slightly larger than the temporary window. Two grooves about 0·5 mm. wide are cut at right angles near one end of the rectangle (Fig. 8). The purpose of these will be described later. The grooves marking the rectangle are now deepened as before, but the final cut through the inner table is checked at the front end of each groove so that four thin bridges of bone are left. An impression is now taken of the upper surface of the skull. Impression materials which require heating, or contain irritants such as clove oil, must be avoided for obvious reasons. The most satisfactory material is a cold-setting cream which has recently become available.[1] A small quantity of this material is mixed with a spatula in a small rubber bowl and from this a container consisting of

[1] "Three in One D.P. Cream," generously provided by the Dental Perfection Company, Glendale, California.

a common metal screw bottlecap, measuring about 2 cm. in diameter by 1 cm. deep, with a central hole about 0·5 cm. diameter, is filled.

After a small quantity of paste has been worked into the grooves in the dry skull with the forefinger, the cap and its contents are inverted over the site. When the paste has set the excess is removed with a blunt scalpel and the metal cap containing the impression is gently lifted clear, examined under the dissecting microscope and, if satisfactory, placed in cold water. A spare impression is desirable.

The operation site is washed out with saline and fragments of impression material are carefully removed. The field is then dried, the bridges of inner table are cut through and the bone flap is detached and preserved. The operator proceeds to scrape clean the cut edges of the inner table, making certain that no impression material remains between the dura and the bone. Four holes for retaining wires are drilled as already described, but the median pair are not used at this stage. Instead, two extra holes are drilled in the left temporal crest. The temporary flat window is inserted over the intact dura and fixed with temporary silver wires threaded through the two temporal crests. The skin incision is then closed.

Meanwhile the assistant wipes excess of water from the impression and gently presses the bone flap into it, the grooves in the latter serving to orientate and centre it in the impression. The impression, plus bone, is now invested with dental stone, using a fine brush and removing bubbles by vibration, and inverted over one-half of a dental inlay flask which has been filled with stone. When the stone has set the impression is removed, leaving the bone flap embedded in the stone.

The grooves left by the impression are packed with dental wax, which has been made plastic by warming, and smoothed off so that the surface of stone, plus bone, plus wax, corresponds with the outer contour of the intact skull.

After investing the surface with a sheet of dental tinfoil, the upper half of the flask is completed in the usual way, and, when setting is completed, removed, together with the tinfoil, from the lower half. The bone and wax are now removed from the lower half of the mould, which is left to dry in a hot air oven for 15 minutes. A piece of thin gold foil cut to the exact shape of the lower surface of the bone flap is then laid in the cavity and pressed home with a pledglet of cotton wool. If this step is omitted water will seep into the acrylic dough and cloud the window. "Liquid tin foil" is not a satisfactory substitute and the cost of the gold foil is negligible. The rest of the surface of the lower mould is invested with tin foil, and the mould is now ready for packing. The window is made from clear acrylic monomer-polymer dough,[2] which is mixed according to instructions. A small quantity of dough,

[2] "Stellon, clear," Amalgamated Dental Co., Melbourne.

flattened and cut roughly to size with a dental spatula, is pressed into the aperture over the gold foil and the upper half of the flask is clamped in position with a powerful spring clamp. The flask is transferred to a beaker of cold water which is brought to the boil in 30 minutes. Boiling must be continued for at least an hour to remove the last traces of the highly irritant liquid monomer.

The flask is cooled in water and opened, leaving the window embedded in the lower half if possible. The upper surface of the window is smoothed with fine emery paper and polished on a piece of velvet. The window is now removed from the mould. After carefully removing the gold foil with a dental pick the lower surface of the window is polished. This is best done with cotton wool on a match stick revolving in a straight dental handpiece, using first emery flour and water and finally buffing with dry cotton wool. An optically-perfect surface is not necessary because the cerebral vessels will be separated from the window only by a very thin layer of cerebrospinal fluid. Finally the edges and lugs of the windows are scraped free from fragments of stone or tinfoil. The final appearance of the window is shown in Fig. 12.

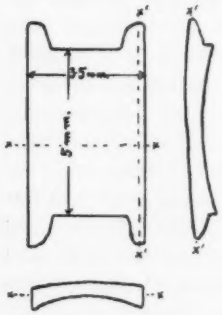

Fig. 12. Completed moulded acrylic window showing approximate shape and dimensions.

When the window is ready the rat is again fully anaesthetized, the incision reopened and the temporary window and wires are removed, the latter being replaced by gold wire. After checking the fit of the permanent window, the dura is incised and reflected. The window is now carefully inserted and wired in. Four shallow grooves may be carefully made in the lateral surface of the window adjoining the bone. In chronic experiments new bone tends to grow into these grooves, thus locking the window in position. The incision is now closed.

Advantages and drawbacks. The main advantage of moulded windows lies in the fact that deformity of the vault of the skull is minimal or absent at the time of insertion. Slight secondary deformation, however, occurs in time, as the cut edge of the inner table becomes rounded. On the other hand, the technique is so intricate and time-consuming that its use seems justified only in experiments in which swelling of the brain is expected.

Operative mortality and complications.

In a series of 293 consecutive experiments with flat or moulded perspex windows 22 rats died during the operation, either from pulmonary atelectasis, caused by excessive secretion of mucus, or from respiratory failure when the window was pressed in. With these exceptions the rats recovered remarkably quickly and usually appeared normal on the following day. Local subdural,

subarachnoid or subpial haemorrhage, caused by operative clumsiness, was not uncommon, but in most cases the blood rapidly and completely disappeared. In early experiments low-grade sepsis occasionally obscured the window without apparently affecting the rat, but this was later avoided by sterilising the instruments after use and adding a little penicillin to the irrigating fluid. Concentrated solutions of penicillin must not be applied to the exposed brain. Rigid aseptic technique is unnecessary.

Normal appearances.

Perspex windows afford a very satisfactory view of a relatively large area of the surface of the brain (Figs. 14, 15). The clarity of the vascular picture is enhanced by the absence of convolutions on the surface of the rat's brain. The

Fig. 14. Freshly-inserted moulded acrylic window showing position and method of fixation. ×5. Reprinted from Byrom (1954), Lancet ii, p. 201.

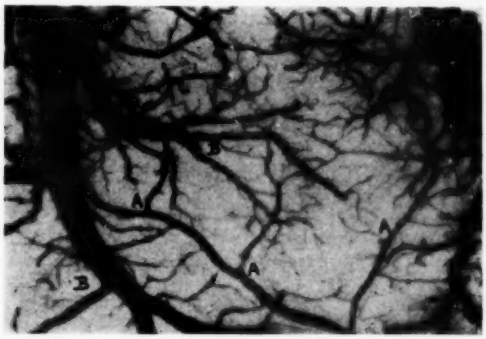

Fig. 15. Appearance of surface of brain as seen through a moulded acrylic window 12 weeks after insertion. (A) Branches of middle cerebral artery; (B) tributaries of sagittal venous sinus. There is no evidence of reaction to the window and the vascular pattern is free from distortion. ×16. Reprinted from Byrom (1954), Lancet ii, p. 201.

arteries seen consist of one or more major branches of the middle cerebral artery and smaller branches of the anterior cerebral artery. These divide and subdivide to form terminal arteries (or arterioles) which continue for a variable distance on the surface and then bend abruptly at right angles to disappear vertically into the substance of the brain. The veins are wider and flatter than the arteries and drain into one or more tributaries of the superior longitudinal sinus. At interscections the artery is usually superficial to the vein. The blood in the two vessels does not differ in colour in the anaesthetized rat.

Applications.

The preparation is well suited to the study of the response of the cerebral vessels to experimental procedures such as the injection or local application of convulsant and vasoactive agents, the response of the leptomeninges to damaging agents, the absorption of blood from the subarachnoid space, and possibly the circulation of the cerebrospinal fluid and the patency of the perivascular spaces. In such experiments it is advisable to allow an interval of two or more weeks to elapse after insertion of the window. If access to the leptomeninges is desired small holes with accurately fitting perspex plugs or screws can be incorporated in the window.

Permanent photographic records can be obtained with a camera attached to one ocular of the microscope. The photographs shown were taken in a darkened room with a 35 mm. camera at a magnification of 5 diameters on the negative, using a hooded 60 Joule electronic flash bulb mounted about 3 cm. above the field and synchronized with the shutter. Prints from such negatives may be enlarged to an overall magnification of 50 diameters without loss of definition. The same conditions are suitable for colour photographs.

Electronic flash illumination is desirable because movement and vibration of the field cannot be eliminated.

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ANTIBACTERIAL ACTIVITY IN MEMBERS OF THE HIGHER FUNGI

II. PSALLIOTIN, THE ANTIBIOTIC OF *PSALLIOTA XANTHODERMA*.

by NANCY ATKINSON

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The antibiotic activity of the edible mushroom *Psalliota xanthoderma* was described by Wilkins (1946) and Atkinson (1946, 1949), who gave a number of characteristics of the antibiotic substance but failed to concentrate or purify it on account of its haphazard lability. Recently Atkinson (1954) found that light inactivated crude mushroom extracts. The haphazard lability of the antibiotic activity in the earlier work was thus most probably due to variations in the degree of exposure of extracts to light. The present paper records the experimental work on light sensitivity of the antibiotic in crude extracts and on paper chromatograms.

On account of its light sensitivity in crude extracts, the antibiotic of *Psalliota xanthoderma* is considered to be new and unique, and, for convenience, the new name psalliotin is suggested for it.

METHODS.

Preparation of extracts. The mushrooms were collected in the autumn of 1954 when a good crop appeared in and around Adelaide. The antibiotic extracts were prepared by crushing up mushroom stems in a little water and squeezing out through gauze. Being unaware at that time of the light sensitivity of the antibiotic in these crude extracts, the whole process was carried out in bright daylight on the bench. Consequently, some extracts were more active than others, depending on the length of time of exposure to light during preparation. Fortunately some extracts were made quickly and immediately frozen in a deep-freeze unit which kept them dark most of the time. Previous work had suggested that frozen or refrigerated extracts maintained activity better than others and, therefore, as a routine, extracts were stored frozen or refrigerated. Actually the preservation of activity was due not so much to the cold as to the exclusion of light. Some mushrooms were also stored hard-frozen. Later, when the light sensitivity of crude extracts had been discovered, extracts were made in a black cabinet in a darkened room with limited illumination with yellow light.

Tests for antibiotic activity. The test organism was *S. typhi* 876, which had been used in previous work and was very sensitive to the antibiotic. Agar containing *S. typhi* 876 (4 ml. of 7-hour broth culture in 100 ml. agar) was poured into petri dishes and the material to be tested was dried on paper which was placed on the surface of the agar. The dishes

were incubated for 18–24 hours at 37° C. and then examined for zones of inhibition of growth around the test papers. Extracts and other solutions to be tested were dried on 13 mm. or 6 mm. paper discs. Paper chromatograms and spots were tested in narrow strips 6–8 mm. wide cut from the full length of the chromatogram or the spot and laid on the surface of the test plates.

Paper chromatography. A spot of 100 µl. of crude extract was dried in air at room temperature on one end of a strip of Whatman No. 1 filter paper which was then run at room temperature (20–23° C.), either ascending or descending, in 80 p.c. alcohol and dried at room temperature in air.

For extraction of the antibiotic, a narrow band of 1–2 ml. of crude extract was dried across one end of a strip of filter paper which was run at room temperature, ascending or descending, in 80 p.c. alcohol and dried in air at room temperature. The position of the antibiotic band was determined by testing a narrow strip cut lengthwise from the centre of the paper. The antibiotic section was then cut off and eluted in water or 80 p.c. alcohol.

After the discovery of the light sensitivity of the crude extracts, all work involving antibiotic material was done either in the dark or with limited illumination with yellow light.

RESULTS.

Extracts from three different batches of mushrooms, 61/54 (from Adelaide), 64/54 (from Cudlee Creek) and 87/54 (from Adelaide), were used at various times. The 61/54 and 64/54 extracts were prepared in daylight, but the 61/54 extracts were made more quickly than the 64/54 extracts and consequently were more active. The 87/54 extract was prepared from a frozen stem and continually protected from light. It was very active. In contrast to the 61/54 and 64/54 extracts, which were brown, the 87/54 extract was pale yellow.

Preliminary work on paper chromatography of extracts 61/54 and 64/54 gave very erratic results. On some papers an antibiotic zone appeared near the solvent front while other papers, apparently identical, showed no zone. Control spots varied similarly in activity. This haphazard behaviour of the antibiotic led to an investigation of the effect of variation in conditions of preparing the spots of crude extract and running the papers. Spots prepared in 2–3 minutes and run for 2–3 hours in an ascending tank in a glass cupboard not facing the window, often showed good antibiotic zones. Similar spots run in a tank by the window failed to show any zones. Control spots kept on the bench beside the tank by the window were also inactive. Other control spots kept in the refrigerator (and thus in the dark) were as active as spots freshly and quickly made, which were invariably active if tested without delay. These results suggested that exposure to daylight might be causing inactivation of the antibiotic. Tests were therefore made on the effect of daylight on the antibiotic in crude extracts and paper chromatograms.

Control spots and spots for chromatography were prepared on filter paper in the shade. The spots were covered until subjected to the test procedure. Ascending and descending chromatograms were run and dried in daylight, others were run in a darkened tank and dried in the shade. Control spots were exposed

to daylight and others were placed alongside in a lightproof box or in a box in the refrigerator. After 3 hours no activity was detected in the spots or chromatograms kept in daylight. Good activity appeared, however, in the tests spots kept in a box on the bench in the light or in the refrigerator. Good antibiotic zones appeared also on ascending and descending chromatograms kept in the dark. These results confirmed the inactivating effect of daylight upon the antibiotic activity of test spots and chromatograms.

To obtain more quantitative results on the inactivation of crude extracts by daylight, 13 mm. discs, soaked in crude extract and dried in air at room temperature (called test discs), were exposed to daylight for various times. Typical results are given in Table 1.

TABLE 1.

Effect of daylight on the antibiotic activity of crude extracts of *Psalliota xanthoderma* on paper.

Extract no.	Time of exposure to daylight	Diameter of antibiotic zone including 13 mm. disc
61/54 1st ex.	3 hr.	trace only.
	5 hr.	no zone
	nil (in box 5 hr.)	23
	nil (in frig. 5 hr.)	23
61/54 2nd ex.	50 min.	17
	2 hr. 23 min.	trace only.
	3 hr. 42 min.	trace only.
	nil (in box 4 hr.)	24
	nil (in frig. 4 hr.)	23
87/54 1st ex.	1 hr. 17 min.	19
	2 hr. 23 min.	14
	3 hr. 45 min.	trace only.
	6 hr.	trace only.
	nil (in box 4 hr.)	27
	nil (in frig. 4 hr.)	27

Thus, in about one hour, daylight greatly reduced the antibiotic activity of test discs of extracts 61/54 and 87/54, and in about 3 hours almost completely destroyed their activity. Similar discs showed no loss of activity when kept either on the bench in a box beside the exposed discs or in the refrigerator in the dark. Crude extracts exposed to daylight either in thin layers in petri dishes or in small volumes in small test tubes were also inactivated by daylight. Thus no activity was detectable on test discs made from 0·5 ml. of 61/54 1st extract exposed in a small test tube for 5 hours to daylight. Test discs of the same extract in a similar tube kept in the dark gave a zone of 22 mm. 87/54 1st extract, exposed in a thin layer to daylight, was almost completely inactivated in 25 minutes and no activity was detectable after 2 hours.

The effect of light of various colours upon the antibiotic activity of crude extracts was investigated by simultaneously exposing test discs to daylight under various Wratten filters. At the same time, and in the same position, control discs were exposed to daylight and others were kept in a box. Typical results are given in Table 2.

TABLE 2.

Effect of light of various colours on the antibiotic activity of crude extracts of *Psalliota xanthoderma* on paper.

Wratten filter used	Time of exposure	Diameter of antibiotic zone, including 13 mm. disc.	
		61/54 2nd ex.	87/54 1st ex.
A (light red)	3 hr.	27 mm.	32 mm.
B (green)	3 hr.	29 mm.	31 mm.
C (dark blue)	3 hr.	27 mm.	31 mm.
D (dark violet)	3 hr.	trace only	20 mm.
E (orange)	3 hr.	28 mm.	30 mm.
F (deep red)	3 hr.	28 mm.	32 mm.
G (orange yellow)	3 hr.	28 mm.	31 mm.
H (pale blue)	4 hr.	26 mm.	31 mm.
K1 (pale yellow)	4 hr.	25 mm.	31 mm.
none (daylight control)	3 hr.	trace only	20 mm.
none (in box, dark control)	4 hr.	26 mm.	31 mm.

Thus all the Wratten filters except D (dark violet) protected the antibiotic activity from inactivation by daylight. The D filter transmitted violet and ultraviolet.

The effect of short wave ultraviolet on the antibiotic activity of crude extracts was investigated by exposing test discs for various periods at 20 cm.

TABLE 3.

Effect of ultraviolet (mainly 250 mμ) on the antibiotic activity of crude extracts of *Psalliota xanthoderma* on paper.

Extract No.	Time of exposure to U.V.	Diameter of antibiotic zone including 13 mm. disc
61/54 2nd ex.	15 min.	17
	33 min.	16
	63 min.	16
	89 min.	trace only
	nil (under cover)	21
87/54 1st ex.	15 min.	25
	30 min.	23
	70 min.	18
	3 hr.	trace only
	nil (under cover)	27

distance from an ultraviolet source of the sterilamp type emitting at least 86 p.c. in the 250 mμ. region with very little emission of longer wave lengths. The lamp was coiled up to give a compact source of ultraviolet and had a filter to remove any visible light. Typical results are given in Table 3.

Thus the short wave ultraviolet, as used here, greatly reduced the activity of test discs in one hour, and almost completely destroyed their activity in about one and a half hours to three hours, depending on the activity of the disc. 87/54 1st extract was exposed to the ultraviolet in a thin layer and tested at various times by removing a portion from which test discs were prepared. This extract was inactivated in less than two hours and somewhat reduced in 20 minutes.

As the yellow Wratten filters protected the antibiotic activity and gave good light transmission, a source of yellow light was sought for general experimental work. A Philips yellow darkroom globe was a readily available source of artificial yellow light. Test discs were exposed inside a black box to this yellow light 20 cm. away and control discs were kept in a box also under the light. Other controls were kept in the dark in the refrigerator. No reduction in activity occurred with test discs of 87/54 1st extract or 61/54 2nd extract exposed up to six hours under the yellow globe. Thus this artificial yellow light was suitable for experimental work with the antibiotic. All further work was therefore conducted in this yellow light in an otherwise darkened room, or in the dark. Under these conditions good antibiotic zones were always obtained from active extracts either on test discs or on paper chromatograms. The average Rf value for the antibiotic in ascending chromatograms in 80 p.c. alcohol at 20–23° C. was 0·76.

Using paper chromatography, preliminary attempts have been made to purify the antibiotic from crude extracts 61/54 and 64/54 prepared in daylight. Water or 80 p.c. alcohol readily eluted the antibiotic from the active section of the paper. It was accompanied, however, by an inactive white crystalline material. The active eluates, which were yellow, also produced amorphous yellow globules when dried. These residues were soluble in water and retained their antibiotic activity. The best eluate (23/8E) so far obtained gave 28 mm. zones on test discs, as compared with 21 mm. zones from the extract 61/54 from which it was prepared.

The effect of daylight upon this partially purified extract 23/8E and the partially purified antibiotic on paper chromatograms was investigated. Test discs of extract 23/8E were exposed to daylight alongside similar test discs in a box. Typical tests gave zones of 28 mm. for the test discs in the box and 14 mm. for discs exposed 3 hours to daylight. The sensitivity to daylight of the antibiotic zone on paper chromatograms was tested by preparing chromatograms with good zones, cutting them in half, and exposing half to daylight while keeping the other half in a box alongside. Typical tests with extract 61/54

showed good zones on the half kept in the box, but no zones were detectable on the half exposed to daylight for one hour, thirty minutes. These results showed that the partially purified antibiotic was still sensitive to daylight.

DISCUSSION.

The antibiotic of *Psalliota xanthoderma* appears to be a new type on account of its sensitivity to light. For convenience the new name psalliotin is suggested for it. The light inactivation of the partially purified extract 23/8E, and of the antibiotic sections of paper chromatograms, suggested that the light sensitivity might well be an intrinsic property of psalliotin itself. However, some sensitizing substance might accompany it on the chromatogram and be responsible for its light sensitivity.

Earlier work on psalliotin was hampered by the haphazard lability of antibiotic preparations which, doubtless, depended upon the extent of exposure to light. The discovery of the sensitivity of psalliotin to daylight and its stability in dim yellow light, as recorded in this paper, has allowed experimental work on this antibiotic to proceed satisfactorily. Much of the earlier work has already been repeated and is being extended. As a result, other interesting properties of psalliotin have been found. It proved unusually stable at moderate temperatures and was inhibitory on agar to various strains of bacteria resistant to most of the commonly used antibiotics. Some of these other characteristics of psalliotin and its antibacterial spectrum will be recorded in a separate paper.

SUMMARY.

The antibiotic activity of crude extracts and partially purified preparations from *Psalliota xanthoderma* was lost by exposure to daylight, to short-wave ultraviolet, or to daylight passing through the deep violet Wratten filter D. It was not inactivated by the yellow light from a Philips darkroom globe, which is therefore recommended for experimental work.

The new name, psalliotin, is suggested for the antibiotic of *Psalliota xanthoderma*.

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THE OCCURRENCE OF GENISTEIN IN SUBTERRANEAN CLOVER (*T. SUBTERRANEUM*, L.) AND OTHER TRIFOLIUM SPECIES

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Subsequent to the recognition of a breeding problem in sheep grazing on pastures dominated by the early-maturing Dwalganup strain of subterranean clover (Bennetts, 1944; Bennetts, Underwood and Shier, 1946), and the implication that an oestrogenic substance was responsible, the isoflavone genistein was isolated by Bradbury and White (1951). More recently, Curnow (1954) has demonstrated that genistein occurs in substantial concentrations in this plant.

In view of the large number of recognized strains of subterranean clover, it was decided to examine most of the available material for genistein. The discovery of a strain in which genistein was absent (or almost so) would be of considerable interest, both genetically and agronomically, and might materially contribute to the solution of the clover-induced infertility problem in sheep.

Little is known of the oestrogenic activity of clovers other than *T. subterraneum*, apart from the discovery of slight activity in one sample of strawberry clover (*T. fragiferum*) by Robinson (1949), and fairly high activity in a number of samples of red clover (*T. pratense*) by Legg, *et al.* (1950). Alexander and Watson (1951), on the other hand, found no oestrogenic activity in the three clovers *T. repens*, *T. fragiferum* and *T. pratense* on feeding fresh plant material to guinea-pigs. Examination of a wider range of species seemed desirable, primarily as an exploratory study within the genus.

METHODS.

All species and strains were grown as spaced single plants, either at the C.S.I.R.O. Field Station "Glen Lossie," Kojonup, or at the Institute of Agriculture, Perth, during 1952 and 1953. Adequate applications of phosphorus and zinc fertilizers at Kojonup and phosphorus and potassium at Perth ensured normal plant growth.

From 20 to 30 single plants of each strain (or species) three were selected at random for analysis. Leaf samples were collected during the first fortnight in September, using a constant area of fresh leaf tissue—400 sq. mm. per plant. The quantities of leaf involved were small, and the above procedure greatly simplified the field work.

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TABLE 1.

The concentration of genistein in the leaf fraction of various strains of subterranean clover.

Australian Strains	*Ratings for Genistein Concentration	Introductions from Overseas (*C.S.I.R.O. Accession Nos.*)	*Ratings for Genistein Concentration
Bacchus Marsh	+++	C.P.I. 9756	++++
Bass	++++	C.P.I. 12451	+
Baulka Maugh	+++	C.P.I. 12679	+++++
Bena	++++	C.P.I. 12683	+++++
Benalla	+++	C.P.I. 12700	+++++
Berlin	+++	C.P.I. 12703	+++++
Burnerang	++++	C.P.I. 12709	+++
Burnley	+++++	C.P.I. 12711	++++
Casterton	++++	C.P.I. 12869A	+++
Clare	++	C.P.I. 12869B	++++
Cranmore	+++	C.P.I. 12869C	++++
Crawley	+++	C.P.I. 12869D	++++
Daliak	++	C.P.I. 12869E	++++
Derrinal	++	C.P.I. 14110(a)	++++
Dwalganup—normal	+++	C.P.I. 14110(b)	++++
Dwalganup—white-seeded	+++	C.P.I. 14110(c)	+++++
Dwalganup—tetraploid	+++	C.P.I. 14194	++++
Edenhope—A	++	C.P.I. 14195	++
Edenhope—B	++	C.P.I. 14196	++++
Flinders	++++	C.P.I. 14197	++++
Four-leaf	++++	C.P.I. 14198	++++
Geraldton	++	C.P.I. 14204	++++
Gingin	+++++	C.P.I. 14206	++++
Hexham—hairy-stemmed	++++	C.P.I. 14211	++++
Hexham—smooth-stemmed	++++	C.P.I. 14213	++++
Hill's Small	+++++	C.P.I. 14215	+++++
Horsham	++	C.P.I. 14217	+++++
Israel	++++	C.P.I. 14218	+++
Kilmore	++++	C.P.I. 14220	++++
King's Park	+++++	C.P.I. 14326(b)	++++
Kyabram	++++	C.P.I. 14326(c)	+++
Kyneton	+++++	C.P.I. 14326(d)	++++
Lake Widgeon	++++	C.P.I. 14326(e)	++++
Liege	+++++	C.P.I. 14328(a)	+++
MacArthur	++	C.P.I. 14328(c)	+++
Malta	+++++	C.P.I. 14381	++++
Mansfield	+++	C.P.I. 14453	++++
Merino	+++	C.P.I. 14454	+++

* The following scale has been adopted:—
++ *c* 2·5 ug./20 sq. mm. leaf. +++ *c* 3·75 ug./20 sq. mm. leaf. ++++ *c* 5·0 ug./20 sq. mm. leaf. +++++ > 5·0 ug./20 sq. mm. leaf.

TABLE 1—*continued.*

The concentration of genistein in the leaf fraction of various strains of subterranean clover.

Australian Strains	*Ratings for Genistein Concentration	Introductions from Overseas (*C.S.I.R.O. Accession Nos.*)	*Ratings for Genistein Concentration
Milton	++++	C.P.I. 14456	++++
Mt. Barker—normal	+++	C.P.I. 14744	+++
Mt. Barker—amber-seeded	++	C.P.I. 15077	+++++
Mt. Barker—white-seeded	+++	C.P.I. 15080	+++
Mt. Barker—tetraploid	++++	C.P.I. 15167	+++
Mulwala	++++	C.P.I. 15257	+++++
Murch	++++	C.P.I. 15259	++
Muresk	+++++	C.P.I. 15260	++++
Nangeela	++++	C.P.I. 15262	++
Northam A	++++	C.P.I. 15263	++++
Northam B	++++	C.P.I. 15264	++++
Northam C	+++++		
Northam D	++++		
Pahantamu	+++		
Philip Island	++++		
Pink-flowered	++++		
Port Fairy	++		
Portugal	+++		
Red-leaf	++		
Rouen	++++		
Ruakura (1932)	++++		
Ruakura (1934)	++++		
Samaria	+++		
Seaton Park	+++++		
Smeaton	+++		
Springhurst	++++		
Tallarook	++++		
Tallarook—tetraploid	++++		
Turkish	++		
Wenijup	++++		
Williams	+++++		
Wodonga	+++		
Yabba North—amber-seeded	+++		
Yabba North—white-seeded	+++		
Yarloop	++++		
Yea	++		

* The following scale has been adopted:—
++ *c* 2·5 ug./20 sq. mm. leaf. +++ *c* 3·75 ug./20 sq. mm. leaf. ++++ *c* 5·0 ug./20 sq. mm. leaf. +++++ > 5·0 ug./20 sq. mm. leaf.

Genistein was determined by the micro-method of Curnow (1954). The fresh leaf tissue was extracted with ethanol, the fats and chlorophyll separated by partition between aqueous ethanol and benzene, and the remaining mixture separated by butanol-acetic acid paper-strip chromatography. Genistein (R_F=0·9) was detected by the pink colour given with ferric chloride. Standard strips with 2·5 μg. and 5·0 μg. genistein were also prepared and the genistein content of the clovers was roughly estimated by visual comparison.

RESULTS.

Subterranean clover strains.

Altogether, 123 strains were tested, including 74 of Australian origin and 49 from overseas, mostly from the Mediterranean region. A compound with R_F=0·90, and giving a pink-coloured complex with ferric chloride was found in all strains without exception. The amount varied from about 2·5 μg. to slightly more than 5·0 μg. per 20 sq. mm. of leaf area. A list of the strains tested, together with the approximate concentrations of the oestrogen, is given in Table 1.

Other Trifolium *species.*

Of the 17 species examined, 14 showed no pink spot in the chromatograms, although in two of them (*T. incarnatum* and *T. dubium*) a reddish-coloured compound was observed. However, this substance appeared quite distinct from the genistein-ferric chloride complex.

TABLE 2.

The concentration of genistein (or closely related compounds) in the leaf fraction of a number of clover species.

Species of Clover	*Rating for Genistein Content
1. *Trifolium alexandrinum*, L. (berseem clover)	—
2. *Trifolium augustifolium*, L. (narrow-leaf clover)	—
3. *Trifolium arvense*, L. (haresfoot clover)	—
4. *Trifolium campestre*, Shreb. (hop clover)	++ (?)
5. *Trifolium cernuum*, Brot. (drooping flowered clover)	—
6. *Trifolium clypeatum*, L. (oriental clover)	—
7. *Trifolium dubium*, Sibth. (yellow suckling clover)	—
8. *Trifolium fragiferum*, L. (strawberry clover)	—
9. *Trifolium globosum*, L. (globe clover)	++++
10. *Trifolium glomeratum*, L. (clustered clover)	—
11. *Trifolium hirtum*, All. (rose clover)	—
12. *Trifolium hybridum*, L. (alsike clover)	—
13. *Trifolium incarnatum*, L. (crimson clover)	—
14. *Trifolium pratense*, L. (red clover)	++ (?)
15. *Trifolium repens*, L. (white clover)	—
16. *Trifolium spumosum*, L. (bladder clover)	—
17. *Trifolium tomentosum*, L. (woolly clover)	—

* See footnote to Table 1. The sign "—" indicates absence of genistein (or compounds closely related to it).

In three species—*T. campestre, T. pratense* and *T. globosum*—a compound giving a colour similar to genistein was observed. The concentration in one of these (*T. globosum*) was equal to the mean value for the subterranean clover strains, whereas in the other two species the level was slightly lower, though still within the range of *T. subterraneum*. A summary of the results is shown in Table 2.

DISCUSSION.

Earlier unpublished studies by the authors, using the bio-assay method of Robinson (1949), showed that 20 strains of subterranean clover were of approximately equal oestrogenic potency. All known commercial strains were included in this survey. The present investigation has confirmed and greatly extended this finding. The 123 strains tested include the majority of the known strains of subterranean clover, and the fact that the oestrogenic substance genistein (or a compound very similar to genistein) was present in every instance is a finding of no small importance.

The concentrations given for the various strains should not be regarded as precise since, at the best, the method is semi-quantitative only. However, the range of values, 2·5 to 5·0 μg. per 20 sq. mm. of leaf area, corresponds approximately to 0·4 to 0·8 p.c. genistein on a leaf dry weight basis, which is the order of magnitude shown by more critical analytical methods (Curnow, 1954).

An examination was made of the frequency distribution of genistein in the Australian and overseas strains, and also between early, mid-season, and late strains. No significant differences were detected, although the possibility of real effects of this kind cannot completely be dismissed.

Whilst it is assumed that the colour reaction of the chromatograms of subterranean clover was due to genistein, it is possible that closely related chemical compounds may have been present in some cases. Pope *et al.* (1953) isolated biochanin A, the 4′-methyl ether of genistein from *T. pratense*; this compound was shown to possess oestrogenic activity. However, biochanin A and genistein have slightly different R_F values on aqueous acetic acid paper chromatograms (Pope, private communication).

In connection with the sheep infertility problem, there appears to be little prospect of finding a genistein-free strain of subterranean clover, which makes the problem of breeding such a strain extremely difficult. However, a breeding programme involving inter-species crosses, if successful, may well assist the solution of the field problem. Subterranean clover, as a pasture legume, is exceedingly well adapted to the south-west of Western Australia, so that the likelihood of other genistein-free clover species, of which there are a number, playing a similar rôle seems remote.

SUMMARY.

More than 120 strains of subterranean clover were examined for genistein content. All strains, without exception, contained appreciable concentrations of this oestrogen in the leaf fraction. A number of other *Trifolium* species were also examined, most of which were genistein-free. However, the concentration in one species (*T. globosum*) was comparable with that in subterranean clover.

The relevance of these findings to the sheep infertility problem is discussed briefly.

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ELECTRON MICROSCOPY OF SECTIONED BACTERIA

A STUDY OF *ESCHERICHIA COLI.*

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The development within the last few years of satisfactory methods of cutting sections of embedded tissues sufficiently thin for effective electron microscopy has enormously widened the scope of the instrument, and, in particular, has suggested a new approach to the study of bacterial morphology. For whereas the earlier attempts to use the electron microscope to examine the bacterial cell met with only limited success owing to the opacity of most bacteria to the electron beam, and may fairly be said to have been disappointing, it may be anticipated that the application of sectioning methods will prove to be a very valuable technique in the elucidation of bacterial structure. Among the outstanding problems of bacterial cytology, that of the nature of the nuclear apparatus remains a subject of controversy, and may well continue so unless the optical observations can be supplemented and extended beyond the optical limits of resolution. While the use of the electron microscope and suitably thin sections overcomes the resolution difficulty, it must be admitted that the method is not free of limitations, the most serious of these being the absence of any technique analogous to the optical staining methods which allow a more or less specific differentiation of cell components. In spite of this drawback the electron microscope undoubtedly has a great contribution to make, as will be apparent from the results obtained in the preliminary investigation of *E. coli*, reported in this paper, together with those of Chapman and Hillier (1953), who investigated cell division in *B. cereus;* of Bradfield (1945), who examined a staphylococcus and a paracolon bacillus morphologically similar to, but slightly smaller than *E. coli*; and of Birch-Andersen *et al.* (1953), who studied *E. coli.* In so far as the results of these enquiries can be compared, there is a substantial measure of agreement in the findings though interpretation may vary to some extent. It becomes evident that bacteria possess a complex nuclear apparatus much more complex than is suggested by many optical micrographs, of which those of Robinow (1949) may be quoted as an example. On the other hand, DeLamater (1953) claims to have demonstrated optically the existence of a nuclear structure similar to that of higher organisms. His views, however, are not generally accepted. The results of electron microscopy, so far, indicate

points of resemblance between bacterial and higher nuclei, but as it is only too easy to interpret bacterial structures in terms of the known apparatus of other cells, and bearing in mind the lack of specific "staining" methods for electron microscopy, it is necessary to exercise restraint in reaching conclusions.

MATERIALS AND METHODS.

All observations were made on *E. coli* (National collection of type cultures No. 8196) grown on either agar plates, in nutrient broth, or in a synthetic medium containing thiamine as an essential growth factor. The solid media were prepared by the addition of 1 or 2 p.c. agar to the nutrient broth (Oxo Ltd., London, England). The synthetic medium was of the following composition:

2·0 gm. $(NH_4)_2SO_4$; 3·75 gm. KH_2PO_4; 11·25 gm. Na_2HPO_4; 0·2 gm. $MgSO_4 \cdot 7\ H_2O$; 0·01 gm. $MnSO_4 \cdot 4\ H_2O$; 0·01 gm. NaCl; 0·0005 gm. $FeSO_4 \cdot 7\ H_2O$; 1 litre of glass-distilled water. Prior to inoculation, 2 ml. of a 10 p.c. (w/v) solution of glucose sterilized by Seitz filtration and 1 ml. of an $M/10^6$ solution of thiamine were added to each 100 ml. of autoclaved medium.

One-litre bottles, continuously shaken, with free access of air, were used for the growth of liquid cultures. All cultures, both liquid and solid, were incubated at 37° C.

The sources of inocula for all cultures from which the specimens for electron microscopy were prepared were 100 ml. amounts of liquid medium inoculated with cells from 18-hour nutrient agar slants and incubated until the stationary phase of growth was well established (20 hours).

For cultures grown on solid media, 10^{10} or 10^9 bacteria, resuspended in about 0·2 ml. of saline after centrifugation from a liquid culture, were spread over well-dried agar plates. Plates were seeded with 10^{10} cells for short growth period experiments, and with 10^9 cells for long growth periods.

In the case of cultures grown in liquid media 300 ml. amounts were inoculated with approximately 10^8 viable cells per ml. (determined by turbidity measurements). The cultures were harvested towards the end of the lag phase, in the centre of the logarithmic phase, at the beginning of the stationary phase, and 12 to 14 hours after the onset of the stationary phase. These times were derived from previously determined growth curves.

In the case of agar plates the cells were washed off at the appropriate time with fixative, while in the case of growth in liquid media the cells were sedimented by gentle centrifugation, the supernatant medium removed and replaced with fixative. The fixatives used were either Palade's (1952) buffered 1 p.c. osmium tetroxide, or that recommended by Chapman and Hillier (1953), viz., 2 p.c. OsO_4 with 0·8 p.c. NaCl, and the period of fixation varied from 2 to 20 hours. After fixation, the cells were very thoroughly washed by repeated centrifugation from distilled water, and then dehydrated by passage through an alcohol series, during which they were left in 70 p.c. alcohol overnight. The cells were next transferred to a mixture of equal parts of absolute alcohol and methacrylate, the latter consisting of *n* butyl methacrylate with 10 to 30 p.c. methyl methacrylate and 2 p.c. benzoyl peroxide as catalyst to promote polymerization. After two changes in the alcohol-methacrylate mixture the bacteria were transferred to undiluted methacrylate and after several changes of this liquid were put into No. 5 gelatin capsules (previously stored over phosphorus pentoxide) in a desiccator and left in an oven at 47° C. for at least 24 hours, which was sufficient to ensure complete polymerization of the methacrylates.

Before sectioning, the gelatin capsules were removed and the ends of the blocks trimmed to about 0·5 mm. square. The microtome used was of the type described by Hodge, Huxley and Spiro (1953) which uses a glass knife. Sections of 0·05μ or less were used, the thickness

being judged by the colour of the floating sections (Porter and Blum, 1953). Micrographs were taken with a Philips instrument operated at 60 K.V. (unless otherwise stated) and with screen magnification of 10,000 to 20,000 ×.

RESULTS.

The first micrographs obtained were of sections of organisms grown on agar slopes and harvested while actively growing (2 hrs.) and at senescence (20 hrs.). These were fixed in Palade's fluid for 2 hours. It was found that these cells were distended, often to breaking point, by the treatment, an effect reported also by Chapman and Hillier (1953). Their recommended fixing procedure was then tried, but with substantially the same result. However, when broth cultures were used, apparently undistorted cell sections were obtained and it seemed that there was some real difference in this respect between cells grown on solid and in liquid media. Subsequent experience invalidated this opinion, but it remained true that preparations of cells grown on solid media showed marked distension more often than those of cells grown in broth, although the latter sometimes showed this effect, while occasionally an embedding of cells grown on agar yielded undistorted sections. Variations of fixing procedure within the limits stated above seemed to have no appreciable effect upon the

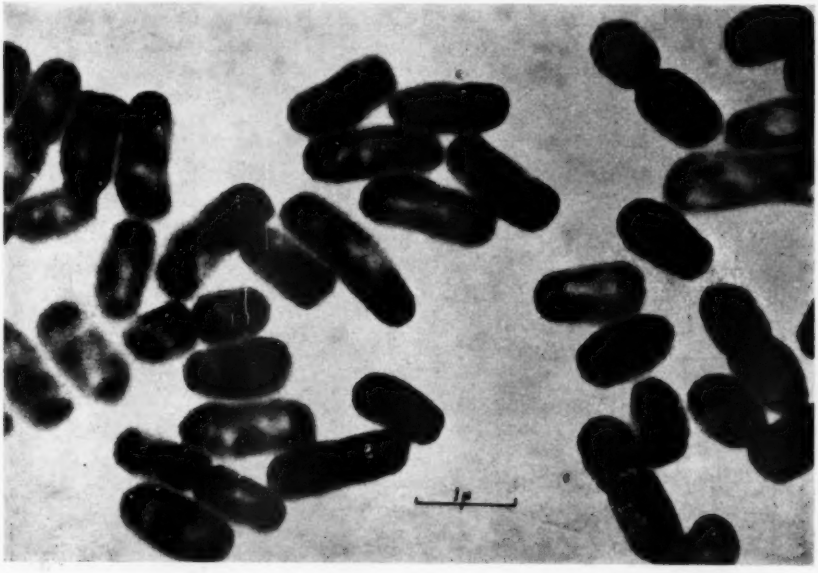


Fig. 1. Whole cells from a 2-hour broth culture of *E. coli*. The cells were dried down on to collodion films from ethanol suspension at the end of the dehydration process. The micrograph was taken at 100 K.V. Magnification, 15,000 ×.

outcome of the experiments. The distension occurred during the polymerization of the methacrylate, for whole cells dried on to collodion at the end of washing, and at the end of dehydration, appeared undamaged and of normal dimensions. No satisfactory explanation of this bursting phenomenon has occurred to us. However, the micrographs of such damaged cells (Fig. 2) are of considerable interest and will be discussed after a consideration of the observations on apparently well preserved bacteria.

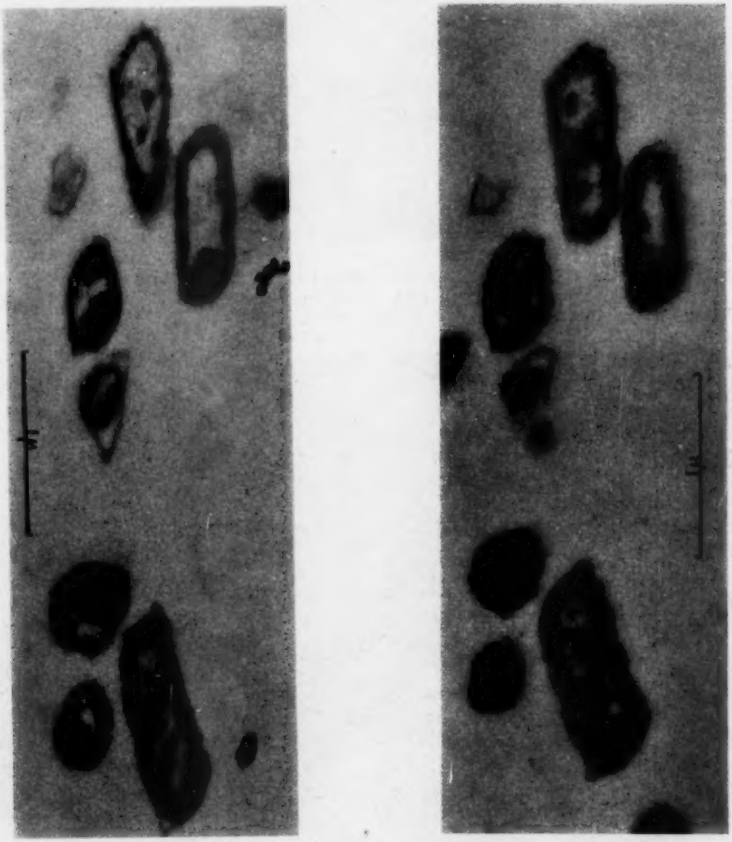
When distension has not occurred there are no appreciable morphological differences between cells grown on agar plates, in broth, or in basal medium, provided that comparison is made of cells taken at corresponding phases of growth.

The conspicuous morphological features revealed by the micrographs of sections is the occurrence of relatively large vacuoles or regions of very low density within the cells, Figs. 2 to 6. Fig. 1 is a micrograph of whole cells fixed in osmium tetroxide, washed and dehydrated, and finally dried down on to collodion films from a suspension in absolute alcohol. Clearly the vacuoles found in the sections are not artifacts of the embedding process, but are present in the bacteria at the end of washing, and at the end of dehydration. The distribution of these vacuoles, both in whole cells and sections, agrees very closely

Fig. 2. Cells from a 20-hour nutrient agar culture of *E. coli*, showing swelling and in some cases bursting of the bacteria during polymerization of the methacrylate. Fixation, 2 hours in Palade's fluid. Magnification, 22,000 ×.

with the distribution of nuclear stains as shown, for example, by Robinow (1949), a conclusion previously reached by Mudd and Smith (1950) from their study of whole cells.

The vacuoles, in sections, are very variable in form and it is not our experience, with the particular organisms studied, that a rigid distinction can be made between the shapes of vacuoles in young dividing cells and in resting cells such as was made by Birch-Andersen *et al.* (1953). We have observed both the large simple vacuole, which they regarded as characteristic of the dividing cell, and also the labyrinthine structure, which they considered typical of resting cells, but we have found both types in both resting and dividing cells.



Figs. 3 and 4. A group of cells from a 20-hour nutrient agar culture of *E. coli*, which have been sectioned at two different levels, showing protrusions of cytoplasm into the central vacuole. Magnification, 35,000 ×.

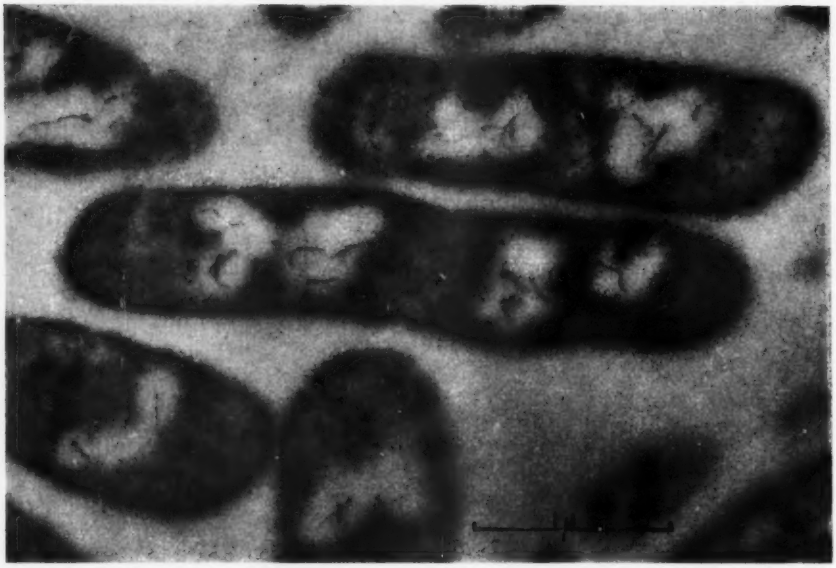


Fig. 5. Sections of growing cells from a 2-hour nutrient agar culture of *E. coli*. Fixation was for 20 hours in Palade's fluid. Slight distension has occurred so that the cell wall is very closely applied to the protoplasm. Magnification, 30,000 ×.

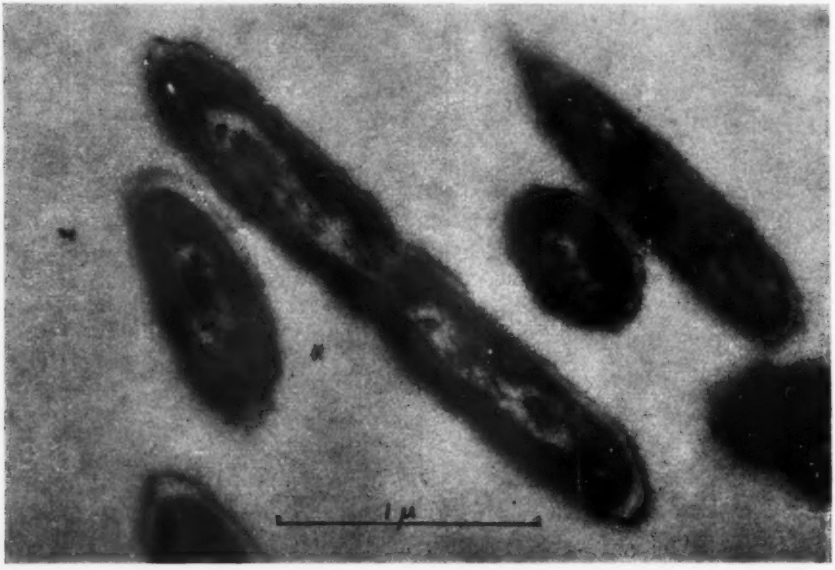


Fig. 6. Sections of dividing cells of *E. coli*, taken at mid-log phase from a culture in synthetic medium. Magnification, 40,000 ×.

In agreement with these authors, and with Bradfield (1954), we have never seen a distinct membrane bounding the vacuole.

Within the vacuoles are found filamentous structures, particularly evident in Figs. 2, 5 and 6, and sometimes larger bodies, Figs. 3, 4 and 6, which are of much the same density as the cytoplasm. In addition there is a fine deposit, probably resulting from precipitation of the vacuolar fluid or gel by the fixative. From Figs. 3 and 4, which represent sections of two different levels of the same group of resting cells, it is quite clear that what may appear as central bodies are very often merely projections of cytoplasm into the vacuoles. This observation does not, of course, preclude the possibility of the occurrence of something of the nature of a nucleolus, but no convincing demonstration of the existence of such structures is as yet forthcoming.

The filamentous structures lying in the vacuoles are particularly well shown in micrographs of cells swollen to bursting point (Fig. 2), but they are clearly not an artifact produced by distension, as they occur in apparently well preserved cells. These threads, which may or may not be circular in cross-section, vary somewhat in width, and in the field from which Fig. 5 was taken widths ranged from about 50 to 200 A.U. The lengths of these filaments cannot be obtained from such thin sections, but it is evident from Fig. 2 that some of them may be at least $0{\cdot}5\mu$ long in resting cells. Beyond recording these observations it is not at this stage possible to do more than speculate about the nature of these fibres. However, it may be remarked that Fig. 2 does suggest that it may be feasible to isolate these filaments for further investigation.

The protoplasm surrounding the vacuole sometimes appears as a homogeneous finely-granular structure, but often it is less uniform and distinct granules may be observed in it. For example, in Fig. 5 the cytoplasm is far from homogeneous and contains granules which are more osmiophilic than the surrounding material. Nothing resembling the mitochondria of higher cells has been found. In general, no distinct cytoplasmic membrane has been observed, but in one case out of many hundreds of cells photographed a very obvious plasma membrane appeared; in all other cases it must be assumed to have remained very closely applied to the underlying protoplasm. In contrast the cell wall usually is very conspicuous (Fig. 6) for it becomes separated from the inner structure during processing. The thickness of the wall may be estimated to be about 100 A.U., while in the single case observed the plasma membrane appeared a little thinner than this. Birch-Andersen *et al.* (1953) record that the fixed cytoplasm shows some orientation perpendicular to the surface, and this we have also found, particularly in cells which have distended during processing.

DISCUSSION.

There is no doubt that the vacuoles seen in sections of bacteria coincide with the less dense areas seen in electron micrographs of whole cells, and that

these are the regions which take up nuclear dyes. But whether this structure should properly be termed a vesicular nucleus is another matter, as has been remarked by Birch-Andersen *et al.* (1953). It is a much more variable structure than the resting nuclei of higher cells, and unless the existence of a surrounding membrane can be established, it is not clear that the same distinction made between nucleus and cytoplasm in other cells is appropriate in the case of bacteria. As to the contents of the vacuoles, it has already been pointed out that apparent central bodies, which one might be tempted to identify as nucleoli, are in fact very often protrusions of cytoplasm, and the nature of the filaments remains to be established. It might be expected that some linear structure must exist to afford genetic continuity and that these filaments may be chromosomes or their analogues. The fact that nothing like a mitotic figure has yet been observed by electron microscopy does not rule out the occurrence of some such process for the sections are so thin that even if such a configuration of the threads occurred it would almost certainly not be detected without a technique of accurate serial sectioning. Since no specific staining technique is yet available for electron microscopy an attempt to isolate the filaments might be worthwhile. Weibull and Hedvall (1953) have examined fractions of disintegrated bacteria without finding anything like these nuclear threads, but their methods of disintegration may have been too violent.

Whatever the true nature of these fine structures, it is clear that the nuclear apparatus of bacteria is much more complicated than the picture based on the earlier results of staining with nuclear dyes. Thus Robinow's (1949) chromosomes would, in fact, appear to be the vacuoles themselves, and the chromosome pattern of cell division which he presents is actually the division and redistribution of vacuoles. If this is so, then the discussion of chromosome behaviour in reproduction, given by Bisset (1950), for example, will need revision; and if the fine threads are indeed chromosomes, a picture of the reproductive process more nearly akin to that in higher organisms may ultimately emerge.

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AN EXPERIMENTAL STUDY OF THE REACTION OF SKELETAL MUSCLE TO INJURY

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The degeneration and regeneration of striated muscle have been studied at length by numerous workers. Useful early observations include those of Waldeyer (1865), Weber (1867), Neumann (1868) and Volkmann (1893). More recent accounts are those of Forbus (1926a and b), Millar (1934), Speidel (1938), Le Gros Clark (1946) and Le Gros Clark and his colleagues (1945, 1947). Speidel made direct observations on muscle fibres under normal and experimental conditions in the living animal; and Le Gros Clark (1946) showed experimentally that under favourable conditions damaged muscle may undergo extensive regeneration.

The object of the present study was to define clearly the extent of regeneration following various kinds of injuries to which muscle is naturally subject, and, especially, to distinguish between regenerating muscle elements and other proliferating cells in the injured areas.

Materials and Methods.

Physical injuries to limb muscles.

Forty-nine experiments were performed on 7 healthy adult guinea-pigs. The muscles selected for study were the rectus femoris, tibialis anterior and extensor digitorum longus of both lower limbs, and the deltoid or lateral head of the triceps on the left side. With the animals under ether anaesthesia, the muscles were exposed and subjected to the following types of injury.

Crush. The muscular tissue of the proximal end of the extensor digitorum longus was completely interrupted by a simple transverse crush, the crushed ends of the muscle being kept in position by the fine connective-tissue framework which still persisted. Crushing was performed by special clamp forceps, the blades of which were smooth and measured 4 mm. in width.

Excision. A small mass of muscular tissue, which measured a few mm. square, was excised from the surface of the distal end of the rectus femoris.

Incision. The muscular tissue of the proximal end of the tibialis anterior was partially transected by means of a fine scalpel. Considerable retraction of the cut ends occurred, but no attempt was made to bring the ends together.

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Burn. A needle which had been heated over a naked flame was thrust into the substance of the deltoid or lateral head of the triceps. The area of damage on the surface was circular in shape and measured about 1 mm. in diameter, and the injury in the muscle extended to a depth of several mm.

The animals were killed 3, 6, 9, 14, 21, 30 and 42 days after operation. The muscles were fixed in 10 p.c. formalin and embedded in paraffin. Sections were routinely stained with haematoxylin and eosin, and usually with phosphotungstic acid-haematoxylin, lissamine-tartrazine, Heidenhain's modification of Mallory's aniline blue method, Sudan black B, and periodic acid and Schiff's reagent, including control sections not exposed to periodic acid. Some sections were also stained by the periodic acid and Schiff technique after digestion with 1 p.c. diastase (B.D.H.) in distilled water for 1 hour at 37° C., and these were compared with sections of rat liver with a high content of glycogen, which were treated in the same way.

Repeated crush injuries to the lingual musculature.

In a further series of experiments, the lingual musculature was subjected to crush injuries of varying intensity. The main purpose of this study was to ascertain the different kinds of degeneration which could be produced in the tongue, and also whether such changes were related in any way to the so-called granular-cell "myoblastoma." Because some authorities consider that this is a degenerative lesion and may result from repeated trauma, the crush injuries were applied at frequent intervals. Four animals were given 3, 5, 6 and 10 crushes, respectively, at intervals of from 7 to 18 days. One animal was killed 4 days, and the others 7 days after the last injury. The sections were stained by the various methods already mentioned.

Chemical injuries.

Five animals were each given multiple intramuscular injections of chemical agents, as follows: (a) ethyl ether, right peroneus longus; (b) absolute alcohol, right tibialis anterior; (c) saturated aqueous solution of pyrogallol, right rectus femoris; (d) 5 p.c. aqueous solution of zinc chloride, left flexor digitorum longus; (e) saturated aqueous solution of potassium permanganate, left tibialis anterior; (f) 6 p.c. aqueous solution of hydrogen peroxide, left rectus femoris. With the animals under ether anaesthesia, 0·1 ml. of the chemical agent was injected into the exposed muscles. The animals were killed 5, 9, 14, 20 and 33 days after operation.

Injection of silica.

A thick aqueous suspension of finely-powdered silica (100 p.c. free SiO_2) was prepared and 0·1 ml. was injected into the deltoid and triceps muscles of the upper extremities of 10 animals. The changes were studied 3, 6, 9, 13 and 18 days, and 4, 5, 6, 9 and 13 weeks after operation.

EXPERIMENTAL.

Results of crush injuries to limb muscles.

Three days after crushing. Many of the muscle fibres at the site of injury have disappeared, the gap being filled with blood and fibrin. Other fibres are necrotic, but a few have retained their endomysial sheath. In some areas the necrotic fibres are invaded by macrophages, which are effecting removal of the damaged tissue, and, in a few places, short segments of tube-like spaces have resulted. These are lined by proliferating fibroblasts and are partly filled with

fragments of necrotic muscle substance and collections of histiocytes. The surrounding cells comprise many histiocytes, and a small proportion of fibroblasts and polymorphonuclear leucocytes (Fig. 1). These infiltrate the necrotic tissue and extend for a variable distance between the individual muscle fibres on either side of the injury. There is no evidence of regeneration during this period.

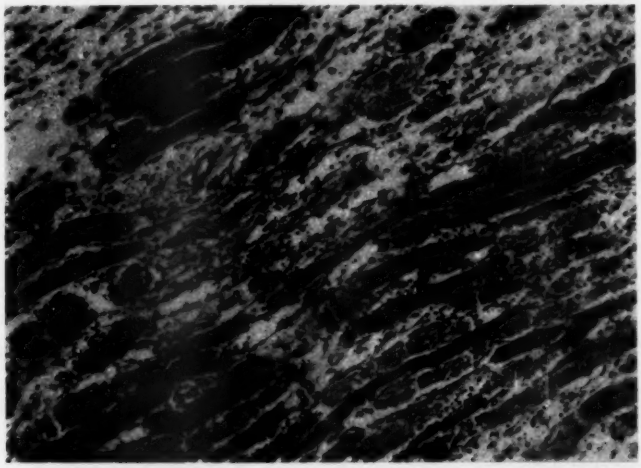

Fig. 1. Crush injury of muscle, third day, to show phagocytosis of necrotic muscle fibres and proliferation of fibroblasts. ×108.

Special staining methods are of value in revealing changes not ordinarily seen with haematoxylin and eosin. After treatment with periodic acid and Schiff's reagent (P.A.S.), some of the muscle fibres adjacent to the necrotic zone, and others more peripherally situated, are found to contain fine red granules which are most numerous in the region of the cross striations. The appearances suggest that the striae have undergone granular degeneration. Many of these fibres, however, show little change in haematoxylin and eosin preparations. Masses of fine red granules are also observed in large numbers of histiocytes. Necrotic muscle fibres are often stained a diffuse dull red and rarely contain granules.

After treating the sections with diastase, the P.A.S.-positive granules in the fibres are no longer obvious; it would appear, therefore, that they are composed of glycogen. Nevertheless, P.A.S.-positive granules, resistant to disastase, are present in many of the histiocytes, and the necrotic muscle fibres still retain their dull red colour. In sections stained with Sudan black B, some of the invading histiocytes and a small proportion of the fibroblasts show small clusters

of specific granules, and the frayed ends of a few necrotic fibres also contain small collections of discrete positive granules. The phosphotungstic acid-haematoxylin method is particularly helpful in distinguishing normal from necrotic muscle. The necrotic fibres stain a brownish red, contrasting well with the blue of the striae of normal or partially damaged muscle. In some areas the necrotic tissue contains many fine blue granules, which often correspond with the faint outlines of the striae; and a similar granular material is present in many of the invading histiocytes. On the other hand, the necrotic fibres are variously stained by the lissamine-tartrazine method. Many are coloured a uniform deep red, others reveal a patchy staining with both lissamine and tartrazine, and a few are predominantly yellow.

This is the place to mention that somewhat similar, but less marked, granular changes were revealed by P.A.S. staining in skeletal muscle removed from a guinea-pig limb which had not been subjected to any form of injury. In some of the fibres there was a little widely-scattered granularity; in other fibres the aggregations were coarse and irregular in outline and sometimes they were connected by fine filaments to form tangled networks in the sarcoplasm (Fig. 13). In these instances haematoxylin and eosin preparations often revealed a little structural change in the form of hyaline degeneration. On the other hand, samples of striated muscle taken from the limb of a guinea-pig embryo revealed no abnormality with periodic acid and Schiff's reagent. Presumably

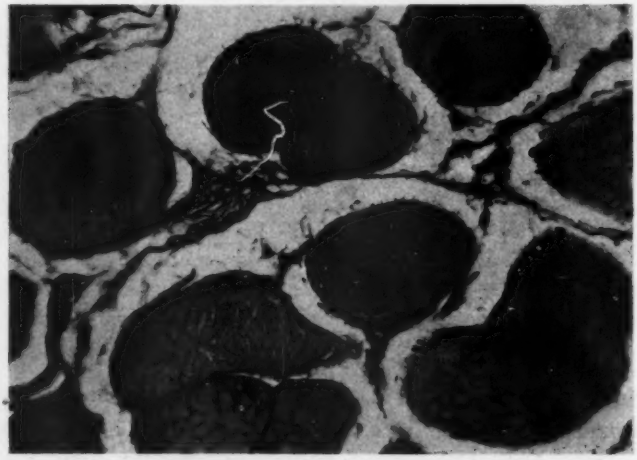

Fig. 13. Transverse section of the limb muscle of a "normal" guinea-pig. Each fibre contains coarse granules and networks of filaments in its sarcoplasm. The muscle was not subjected to any form of injury. P.A.S. ×312.

the adult guinea-pig has been subjected to injury in the course of its normal activities. These findings clearly show that skeletal muscle is very susceptible to minor injuries, and it is important to emphasize that special stains are usually necessary for the detection of the changes in the fibres.

Six days after crushing. The cellular infiltration contains many fibroblasts and histiocytes. Active signs of muscle regeneration are present. Many young fibres have grown out from the stumps of the pre-existing normal fibres and have extended for a variable distance into the cellular and necrotic zone. Often they are seen to have grown along the tube-like spaces which are occupied by histiocytes and fragmented portions of necrotic muscle fibre. The regenerating fibres lie parallel to the long axis of the muscle and, in some places, have met and interdigitated in the centre of the injured area. The majority of the fibres show longitudinal striations and, occasionally, faint cross striations. A few of the young fibres are vacuolated. The muscle nuclei are very numerous at the growing end, but there is no evidence of mitosis.

Nine days after crushing. The zone of injury contains a considerable amount of fibroblastic tissue and many regenerated muscle fibres. A small amount of haemorrhage and necrotic muscle tissue still persists in the centre of the crush and where the fibres have come up against this tissue their growing ends tend to widen out and form multinucleated plasmodial masses. A striking feature is the extreme vacuolar degeneration of some of the pre-existing muscle fibres on either side of the injury.

Fig. 2. Crush injury 6 weeks after operation. There is some disorientation of the regenerated fibres. ×115.

Later stages. From three to six weeks after crushing (Fig. 2) there is muscular union associated with almost complete disappearance of the fibrous tissue. In addition, little cellular exudate remains. The fibres are grossly disorientated, but in some areas they have retained their parallel arrangement. At the end of three weeks, the fibres still show some variation in their staining properties, and have an average diameter of 8μ compared with 23μ of the adjacent normal muscle fibres. Six weeks after operation, many of the fibres are normal in diameter, but others vary greatly in size.

Results of injury by excision and incision.

A cross incision is tantamount to an excision since it creates a gap; and hence both types of injuries are considered together.

Three days after injury. The area between the ends of the muscle fibres contains blood and fibrin, many histiocytes, and a few fibroblasts and polymorphonuclear leucocytes. The muscle fibres at the margin of the injured zone show signs of degenerative changes. They are swollen and vacuolated and some have undergone necrosis and fragmentation. A striking feature is the invasion and phagocytosis of the contractile substance of dead fibres by groups of histiocytes (Fig. 3), and there is proliferation of fibroblasts in the endomysium between these fibres. In this way, cellular tubes containing macrophages and lined by fibroblasts have resulted. The findings with the special staining methods are generally similar to those following crushing injuries (Figs. 4, 5).

Fig. 3. Section through muscle 3 days after local excision. Necrotic fibres are being phagocytosed by groups of histiocytes. ×396.

Fig. 4. Section through muscle 3 days after incision. The small deeply staining cells in the endomysial tubes are histiocytes. Muscle fibres in upper part of field stain strongly with P.A.S. and show diffuse fine granuiarity. ×108.

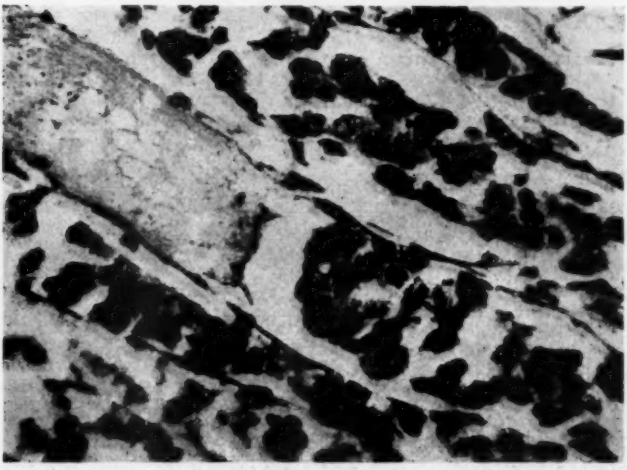

Fig. 5. High-power view of part of Fig. 4. The histiocytes contain granules which stain with P.A.S. The degenerated muscle fibre is vacuolated. ×396.

Six days after injury. A considerable amount of blood and fibrin is still present, but it is invaded by many fibroblasts and new-formed capillaries. Early regeneration of muscle fibres is also evident. New buds, sometimes with expanded growing tips, sprout from the stumps of old fibres and extend into the fibroblastic tissue for a short distance (Fig. 6).

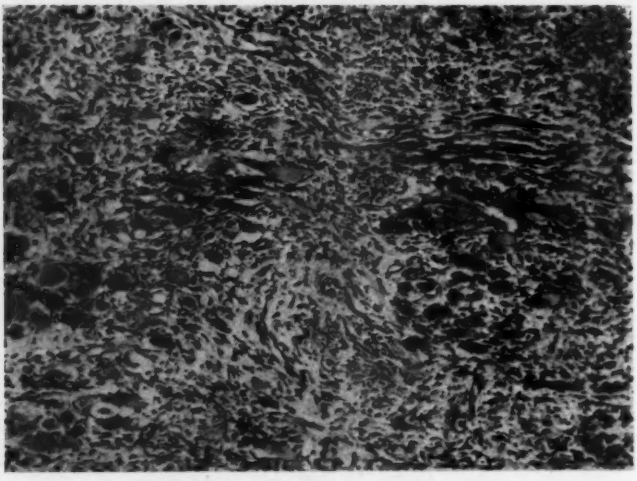

Fig. 6. Incision of muscle, 6 days after operation, showing stumps of pre-existing fibres from which many regenerating fibres extend into the fibroblastic tissue. ×102.

Nine days after injury. Regeneration of muscle fibres has progressed further, but the formation of fibrous tissue appears to act as a mechanical barrier to their extension. The new-formed fibres partly traverse the fibroblastic tissue, but they are disorientated and are disposed in all directions. Giant multinucleated cells of muscle origin form a conspicuous feature and many lie in this fibroblastic tissue where, in a single plane, they appear to be isolated elements. A rare but interesting finding is the presence of new-formed capillaries filled with blood in the sarcoplasm of some regenerating fibres (Figs. 7, 8). No mitoses are present in the young fibres.

Later stages. At the end of 14 days the site of injury is occupied by dense fibrous tissue and regenerating muscle fibres. The young fibres may extend deeply into the fibroblastic tissue and they constitute about one-quarter to one-third of the bulk of the tissue. The later stages show a further increase in fibrous tissue.

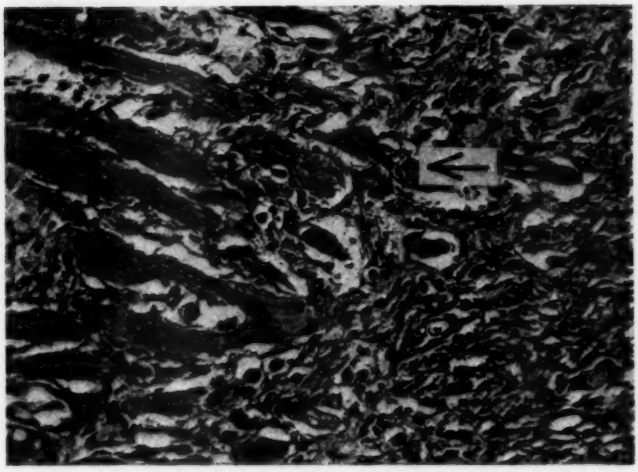

Fig. 7. Site of excision, ninth day, to show a regenerating fibre with new-formed capillary, indicated by the arrow. Note also the collection of nuclei in the fibres nearby. ×174.

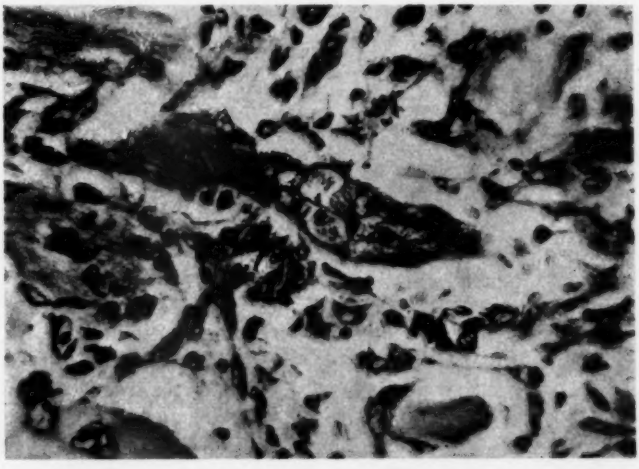

Fig. 8. Enlarged view of the regenerating fibre with capillary indicated in Fig. 7. The lumen of the vessel contains red-blood cells. ×360.

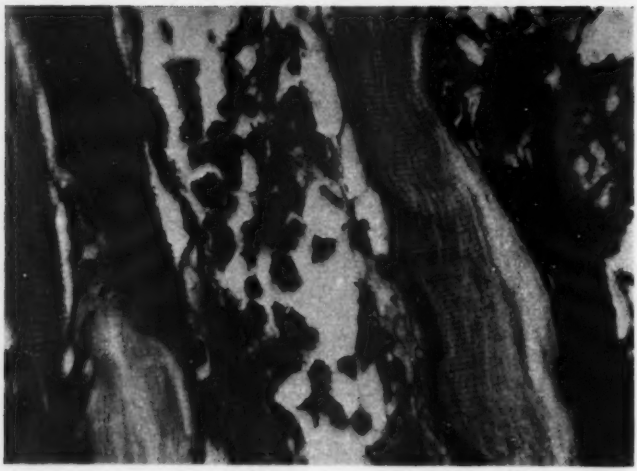

Fig. 9. Deltoid muscle 3 days after a burn, showing granular change limited to the striae of the fibres. The invading histiocytes contain a few granules which are also positive with P.A.S. ×396.

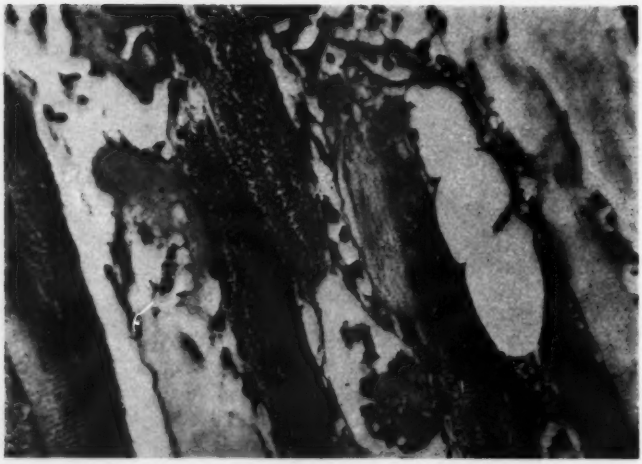

Fig. 10. Granular change in muscle fibres 3 days after a burn, in a more advanced stage than in Fig. 9. The necrotic fibres are non-granular and are being phagocytosed by histiocytes. ×336.

Results of burns.

Three days after burning. The site of injury shows a zone of necrotic muscle fibres. The fibres are swollen, homogeneous, and devoid of nuclei and are undergoing some fragmentation. However, many of these fibres have preserved their contour, and, in addition, some show faint cross striations. Except for an occasional fibrocyte, the endomysial connective tissue in the necrotic area has disappeared. Extending between the muscle fibres, particularly at the junction of normal and necrotic zones, there is an infiltration consisting of many histiocytes and a few polymorphonuclear leucocytes. The sarcoplasm of the injured muscle cells is invaded and replaced by the histiocytes. In a few areas the infiltration extends between the necrotic muscle fibre and the reconstructed endomysium, and in other places the endomysial sheath contains numerous macrophages and remnants of sarcoplasm. The special staining methods reveal changes similar to those following other injuries; these are depicted in Figs. 9–12.

Six days after burning. The cellular infiltration is abundant and vascular, and contains many fibroblasts. A great deal of the necrotic tissue has undergone dissolution, although in some areas many necrotic fibres still persist. There is great regenerative activity of the muscle; many young fibres extend into the cellular zone for a considerable distance from the muscle on either side of it. There are no mitoses in the regenerating fibres. In a few places, where the injury has been minimal, the affected tissue is occupied by numerous young fibres, and the cellular infiltration is relatively slight.

Nine days after burning. The histological picture shows much the same appearance, but now there is much fibroblastic tissue. Mitoses are present in this, but not in the regenerating muscle fibres.

Later stages. At the end of fourteen days the new-formed muscle fibres are widely separated by fibrocellular connective tissue, and constitute a small proportion only of the cellular content of the injured area. Very few necrotic muscle fibres remain, and they are undergoing absorption by investing macrophages and multinucleated foreign-body giant cells.

Results of repeated crushing of lingual muscle.

At all stages the changes are less than was anticipated from the injuries inflicted. In preparations stained with haematoxylin and eosin, the muscle fibres are rather variable in appearance, but there are usually no other obvious changes. However, striking changes are evident in sections stained by the periodic acid-Schiff method. Many of the fibres show a fine red granularity, and, on cross section, the appearances are very much like those of "myoblastoma" (Fig. 14). It is pertinent to note here that, after treatment with periodic acid and Schiff's reagent, some human adult tongues, otherwise presumed to be normal, show similar but less pronounced structural changes; some of the granules are diffuse and fine, others are coarse, and sometimes they appear in

Fig. 11. Burn of muscle, 3 days after injury. The deeply-staining normal muscle fibres contrast with the pale necrotic fibres. Phosphotungstic acid-haematoxylin. ×93.

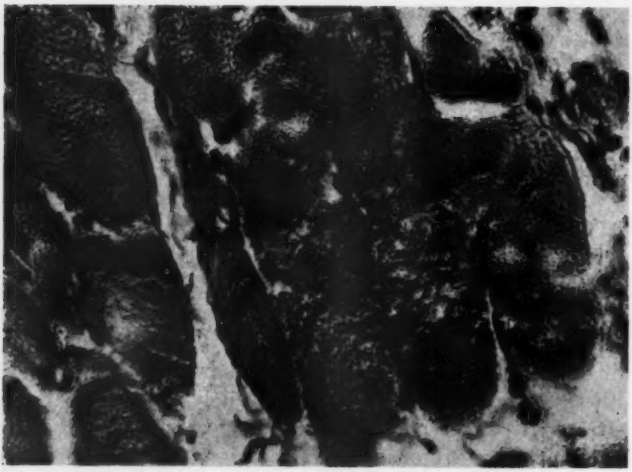

Fig. 12. High-power view of part indicated in Fig. 11 to show the fine granularity in the necrotic fibres. ×336.

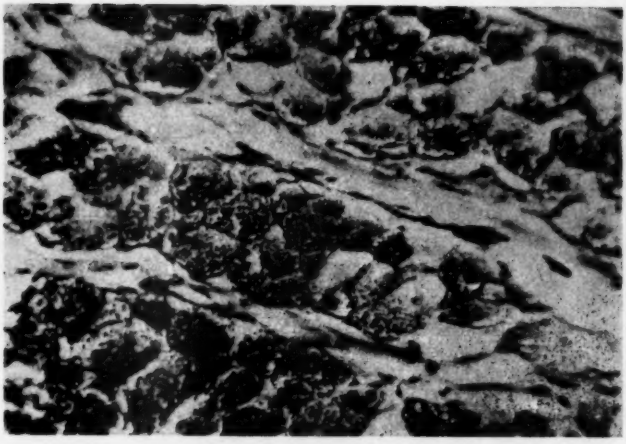

Fig. 14. Transverse section of lingual musculature of a guinea-pig, showing granular change in the fibres following 3 crush injuries. P.A.S. ×312.

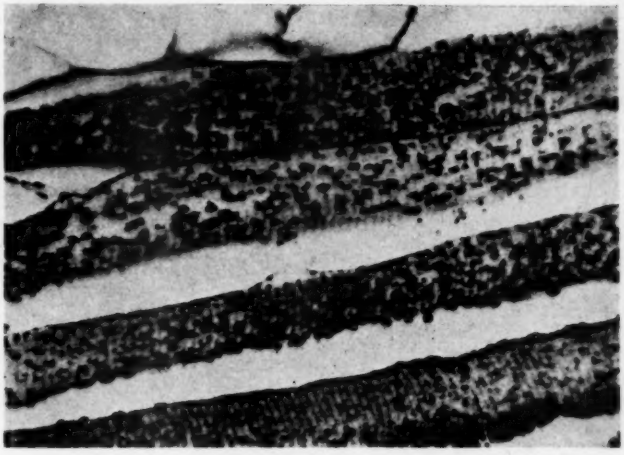

Fig. 15. "Normal" human adult tongue, to show granularity of the muscle fibres. Many of the granules appear as rod-shaped bodies which are no longer than the length of a sarcomere. P.A.S. ×492.

the long axis of the fibre as small rod-shaped bodies, none of which is longer than the length of a sarcomere (Fig. 15). The P.A.S.-positive granules are hydrolysed when treated with diastase.

Results of chemical injuries.

The changes observed in the skeletal muscle in the different forms of chemical injury are very similar to one another and to those following simple mechanical trauma. An extensive area of necrosis develops at the site of injection, and, in injury induced by potassium permanganate, the necrotic fibres also show a strong tendency to fracture transversely across the isotropic discs. At the end of five days, the necrotic area is surrounded by a wide inflammatory zone containing many fibroblasts and also many young regenerating muscle fibres. In the lesion produced by pyrogallol, there are large numbers of macrophages packed with brown pigment granules and a few necrotic muscle fibres also assume a brown colour owing to diffuse pigmentary change. The pigment does not give the iron reaction.

Later stages show the development of a great amount of fibrous tissue in which few regenerating muscle fibres can be seen. Notable in the damage of muscle produced by pyrogallol is the appearance of many foreign-body giant cells at the margin of the necrotic zone. These cells are evident in the 14-day stage and they are actively concerned in the removal of the necrotic fibres (Fig. 16).

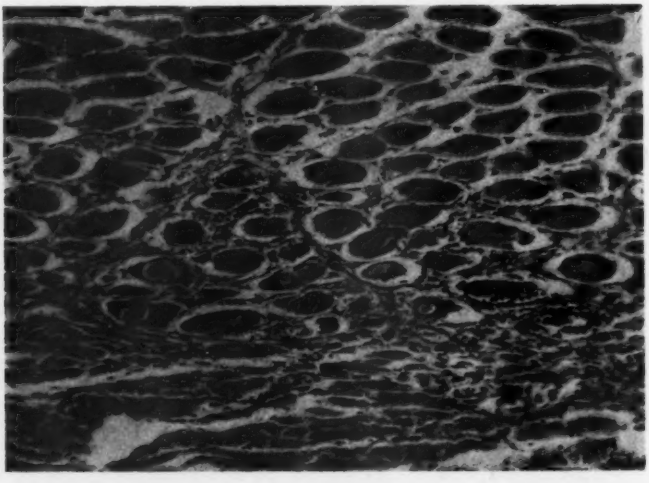

Fig. 16. Phagocytosis of necrotic muscle fibres by foreign-body giant cells 14 days after injection of pyrogallol. The dark staining masses are the giant cells. ×114.

Results of silica injections.

At the end of three days the muscle fibres at the site of injection are replaced by large inflammatory foci which consist of numerous polymorphonuclear leucocytes and other inflammatory cells in various stages of degeneration, closely packed crystals of silica, and a moderate amount of granular material. Many of the neighbouring muscle fibres are necrotic and are separated from one another by oedema and cellular exudate and, in places, the damaged fibres are being invaded and phagocytosed by histiocytes.

In the six- and nine-day experiments the siliceous nodule is surrounded by a wide zone of chronic inflammatory cells, including a few foreign-body giant cells. The lesion is partially enclosed peripherally by a small amount of fibroblastic tissue which is mainly of epimysial origin. The muscle tissue itself shows early signs of regeneration.

In the later stages (Fig. 17) the nodule is seen to be composed almost entirely of foreign-body giant cells and histiocytes. Most of the silica is contained within the cells. A notable feature is the absence of fibrosis, even after thirteen weeks.

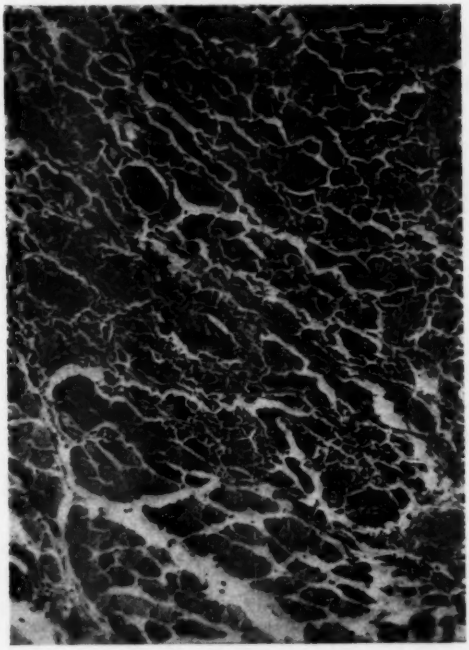

Fig. 17. Siliceous nodule in the deltoid muscle, 4 weeks after injection, to show many foreign-body giant cells and histiocytes. There is no fibrosis. ×120.

DISCUSSION.

The experiments reveal that extensive injuries to skeletal muscle are followed by signs of regeneration after the third day, but that total restoration of the destroyed muscular tissue is usually prevented by the formation of scar tissue. The main change in the first three days is the degeneration of the separated fragmented portions of the muscle and the infiltration of this necrotic tissue by numerous inflammatory cells. These include many histiocytes which invade and phagocytose the necrotic muscle fibres. In this way the damaged fibres are removed and the process results in the formation of tubes containing a variable number of macrophages. These tubes are of short length and lie on either side of the injured zone, and they are lined by proliferating fibroblasts which are interspersed between the necrotic muscle fibres from an early stage. The walls of some of these tubes have collapsed, but most are kept patent by accumulations of histiocytes and isolated fragments of necrotic muscle fibre.

Regeneration first manifests itself as multinucleated outgrowths from the stumps of the old fibres, and many of the new-formed fibres grow into the endomysial tubes *pari passu* with the rapid removal of the necrotic muscle substance. Most of the fibres, however, lie in the cellular granulation tissue which has formed. By the end of the sixth day regeneration is well advanced, the greatest degree of regeneration being evident in the crush injuries where the fibres can be seen growing along the reconstructed endomysial tubes for some distance. The fibres are long and slender and many have tapering processes, but occasionally the growing tip expands into a bulbous mass. The terminal expansion may show longitudinal striations, but the myofibrils are widely separated and irregularly arranged in the sarcoplasm. Nuclei may be present and are usually situated away from the growing end. These plasmodial expansions often appear when the fibres traverse dense fibrous tissue, but they are also seen when necrotic muscle or blood and fibrin lie in the pathway of the regenerating fibres.

Further growth of the new-formed fibres is restricted by the great amount of fibroblastic tissue which now fills the gap between the ends of the injured muscle. The young fibres traverse this fibroblastic tissue and some effect muscular union across the gap. Others terminate as the multinucleated masses, already described, and these may appear as isolated giant cells in the dense fibrous tissue stroma.

The almost complete reconstitution of the muscle following the crush injuries is attributable to the persistence of portion of the endomysial and sarcolemmal framework, as well as to the disappearance of the fibrous tissue. My experiments confirm the important part played by the endomysial tubes in the regeneration of muscle, as stressed by Pfuhl (1937) and later by Le Gros Clark (1946). The latter showed that new endomysial tubes are built up on a scaffolding of the

old necrotic fibres by the rapid proliferation of fibroblasts and that they provide channels along which the young fibres grow freely. In this way, under favourable conditions, the reconstitution of the muscle may be almost complete.

Careful examination of the tissues in all the experiments reveals that regeneration occurs solely as the result of outgrowths from the stumps of the old fibres. There is no formation of independent muscle cells of round and spindle-shaped form as described by some authors (Dawson, 1908-09; Forbus, 1926a and b). However, there is good evidence that a damaged fibre may sometimes give rise to multiple cells by budding, a process well illustrated in the paper by Le Gros Clark (1946, Fig. 23). But this is of rare occurrence, and it is clear that the majority of the cells in the endomysial tubes are non-muscular in origin and are in fact histiocytes. These cells are concerned with the removal of the necrotic muscle substance from an early stage and their intimate association with the damaged fibre may readily give a false impression of a muscular origin.

Recently, Levander (1941, 1945) has stated that the new-formed fibres of regenerating striated muscle develop from connective tissue by a process of metaplasia and not from the old, pre-existing fibres. He considers that this differentiation occurs as a result of induction. No evidence for this was observed in the present study.

The muscle nuclei show a considerable degree of proliferation and are arranged in chains or, occasionally, in small clusters. They are at first centrally placed in the fibre but have moved to a peripheral position by the end of the second week. The new-formed fibres show no evidence of mitotic activity, their proliferation being essentially amitotic. Any mitoses seen in the tissues are confined to the histiocytes and fibroblasts.

Special staining methods are necessary to reveal minimal changes in the muscle fibres, and, in this respect, the periodic acid-Schiff reaction is of particular value in demonstrating structural changes not apparent with haematoxylin and eosin. As already mentioned, the limb muscle of a guinea-pig not exposed to any form of experimental injury, and presumably normal human adult tongues, show a fine granular change with periodic acid and Schiff's reagent, whereas no abnormality is evident with the usual staining procedures. The granular changes revealed by this method in repeatedly crushed lingual muscle may possibly have a bearing on the nature of the so-called "myoblastomas" of the tongue.

SUMMARY.

The results of experimental injuries of various kinds to skeletal muscle have been studied in guinea-pigs.

Skeletal muscle has considerable powers of regeneration, but the degree to which this is manifested is limited by proliferating fibroblastic tissue which

competes with it. Regeneration of muscle fibres is maximal with crush, and minimal with extensive gap-producing injuries. The regenerating sprouts show no evidence of mitotic activity.

Histiocytes in the injured muscle play a rôle in the removal of the necrotic fibres, often filling the endomysial tubes. In the past they have frequently been mistaken for altered muscle fibres, but distinction is possible.

Fibrous tissue formation is usually abundant after injuries to skeletal muscle; but following crush injuries its formation is reversible, and it is surprisingly scanty in the silicotic focus.

Minimal changes, not demonstrable by the usual stains, occur in muscles otherwise presumed to be normal. They have been observed in the limb muscles of guinea-pigs not subjected to any form of experimental injury, and also in human adult tongues.

Repeated crushing of the tongue can produce granular changes in the muscle fibres resembling those of "myoblastoma."

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THE PHYSIOLOGICAL CHARACTERISTICS OF GROUP L STREPTOCOCCI

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Streptococci of Group L have been isolated mainly from dogs and pigs and have not been studied as extensively as the human pathogens of Group A or the animal pathogens of Group B and Group C.

Group L was differentiated by Fry (1939) who found that the colonies were β-haemolytic, the organisms produced soluble haemolysin, fermented trehalose, and sometimes lactose, but not mannitol, salicin, or sorbitol. They did not hydrolyse sodium hippurate or grow on bile agar (Wilson and Miles, 1946). Laughton (1948) described six strains of β-haemolytic Group L streptococci isolated from cases of clinical disease in dogs. These strains fermented lactose, sucrose, salicin, maltose and trehalose, and gave variable results with sorbitol and glycerol. They did not hydrolyse sodium hippurate. Hauge (1950) found that all his twenty-two strains of Group L streptococci fermented lactose, sucrose and trehalose, but not sorbitol, and eleven of them split sodium hippurate. The strains of Group L streptococci previously described have all given β-haemolysis (Fry; Laughton; Hauge; and Collier, 1951).

In the course of an investigation of *viridans* streptococci, a collection of fifty-eight strains of streptococci of varying haemolytic powers, isolated from pigs, was received, and preliminary grouping showed that sixteen of these strains belonged to Group L. Nine of the Group L strains were β-haemolytic, and seven produced haemolysis of the α-type. This paper reports an investigation of the biochemical and physiological characteristics of these Group L streptococci.

Materials and Methods.

Source of strains.

The sixteen Group L strains were isolated from pigs slaughtered during routine killing at a bacon factory; none of the animals showed any clinical symptoms. Strains QJ, QS, Qo, E2, E7 and E8 were isolated from pneumonic lungs, and strains QC, QR, Qa, Qe, E1, E13 and E17 from normal lungs. Strains QH, QI and QP were isolated from the liver, spleen and lung of the same animal which showed no pneumonia but had adhesions of the lungs, pericardium, liver, spleen and intestines. Other organisms such as coliforms and staphylococci were present in all the specimens. Strains SHC 176, SHC 313 and 21203/44 were Group L strains obtained from the Streptococcal Reference Laboratory, Colindale, London.

Antisera, extracts and method for group precipitin tests.

Grouping sera were prepared against strains of Groups A to O, with the exception of Group D, by repeated intravenous inoculation of rabbits with pepsin-treated, formalin-killed vaccines. Group D antiserum was prepared by a slight modification of the method of Shattock (1949), rabbits being inoculated intravenously with organisms shaken for 4½ minutes with Ballotini glass beads, size No. 12, in a mechanical disintegrator (Nossal, 1953).

Extracts for grouping were prepared by the methods of Lancefield (1933) and Fuller (1938).

Group precipitin tests were carried out by carefully layering a small amount of extract on to one or two drops of antiserum in a very small tube. Homologous extracts gave a marked white ring of precipitate at the interface within 5 minutes.

Cultural and biochemical examinations.

Haemolysis was investigated on agar plates containing 10 p.c. horse blood incubated aerobically and anaerobically for 24 and 48 hours.

Fermentation tests were carried out in peptone water containing Andrade's indicator, 1 p.c. of the test carbohydrate and 5 p.c. horse serum. Approximately 4 ml. amounts were inoculated with 2 drops (0·04 ml.) of 24-hour digest broth cultures of the strains to be tested. Smaller inocula did not always grow in salicin and mannitol. The tests were examined every day for 7 days.

The production of soluble haemolysin was tested by growing the strains in 20 p.c. horse serum broth. After 6 hours and over-night incubation 1 ml. of culture was added to 1 ml. of a 5 p.c. suspension of horse red cells in saline and the mixture incubated at 37° C. for 2 hours. Lysis indicated the presence of soluble haemolysin.

Growth in the presence of bile was tested on "ditch plates" (Williams and Hirch, 1950). A diametral incision was made in blood agar plates; half the medium was removed and replaced by agar containing 10 p.c. horse blood and Oxoid Desiccated Ox Bile to give a final concentration of 40 p.c. bile. The strains to be tested were streaked right across the plates, the growth on the blood agar side acting as a control. Two strains of Group D streptococci were also included as controls.

Hydrolysis of sodium hippurate was tested by the ferric chloride method of Ayers and Rupp (Lancefield, 1933). A Group B streptococcus was included as a positive control.

Catalase production was tested on digest agar slope cultures. After incubation at 37° C. for 5 days the slopes were flooded with hydrogen peroxide and observed for the evolution of gas.

RESULTS.

Acid and formamide extracts of the sixteen pig strains gave positive precipitin reactions with Group L antiserum prepared against strain 21203/44. This serum gave strong positive reactions with acid, formamide and enzyme (Maxted, 1948) extracts of 21203/44, SHC 176 and SHC 313, and has given no reactions with strains of any other Group from A to O, or with eighty strains of *viridans* streptococci from human sources. None of the Group L strains reacted with sera of any other group.

The sixteen pig strains were Gram-positive cocci in chains of varying length and none produced catalase.

Strains QH, QI, QJ, QP, QS, Qo, E1, E7 and E8 produced β-haemolysis

TABLE 1.

Physiological characteristics of Group L streptococci.

Strain number	Lactose	Sucrose	Raffinose	Mannitol	Maltose	Salicin	Inulin	Trehalose	Sorbitol	Haemolysis	Soluble haemolysin
SHC 176, SHC 313	—	A	—	—	A	—	—	A	—	β	+
21203/44	A	A	—	—	A	—	—	A	—	β	+
QH, QI, QJ, QP, QS, Qo, E1, E7, E8	—	A	—	—	A	—	—	A	—	β	+
Qa	—	A	—	—	A	—	—	A	—	α	o
QR	A	A	—	—	A	—	—	A	—	α	o
QC, E2, E13	A	A	A	—	A	—	A	A	—	α	o
Qe	A	A	A	—	A	A	A	A	—	α	o
E17	A	A	A	—	A	—	—	A	—	α	o

A = production of acid. — = no acid detected. o = no soluble haemolysin detected. β = clear haemolysis.
α = zone of greening round the colonies.

under aerobic and anaerobic conditions and had glossy colonies. Strain QS produced typical "minute" colonies like those of Groups E, F, G and M.

Colonies of strains QC, QR, Qa, Qe, E2, E13 and E17 were surrounded by zones of α-haemolysis of various sizes when incubated aerobically, and produced similar zones or were non-haemolytic when incubated anaerobically. Strain Qa had typical glossy colonies, but the remaining six strains had flatter colonies of the semi-transparent matt type.

None of the Group L strains grew in the presence of 40 p.c. bile, while the control Group D strains grew well on this medium.

The fermentation reactions are set out in Table 1. If the strains fermented a given substrate they did so within 2 days.

The nine β-haemolytic strains gave the biochemical reactions described by Fry, which were identical with those of the three Group L strains included for reference. Two of the α-haemolytic strains, QR and Qa, gave the same reactions while the remaining five fermented a greater range of carbohydrates. One α-haemolytic strain, QC, hydrolysed sodium hippurate, but the others showed no activity.

With the exception of strain E1, all the β-haemolytic strains were isolated from pneumonic lungs, and all the α-haemolytic strains except E2 came from normal lungs. However, streptococci of the *viridans* type were isolated from a number of other pneumonic lungs and also from normal lungs.

DISCUSSION.

The α-haemolytic Group L strains were more active biochemically than the β-haemolytic strains. This finding confirmed the results of Laughton, who tested six of her twelve Group L strains and found that they fermented salicin in addition to lactose, sucrose, maltose, and trehalose. Although her strains produced β-haemolysis on primary aerobic incubation, only one of the twelve strains produced soluble haemolysin.

In the present work the α-haemolytic strains appeared to be true non-haemolytic forms unlike the Group A strains of Todd (1928) and Fry (1933), which gave α-haemolytic colonies on aerobic plates but produced typical β-haemolytic colonies when incubated anaerobically. Repeated plating of these α-haemolytic Group L strains, both aerobically and anaerobically, failed to produce β-haemolysis, and soluble haemolysin was never detected.

Although the original serological groups contained only β-haemolytic strains (Lancefield, 1933), many were found later to contain non-haemolytic varieties. In Group B nearly half the strains were non-haemolytic, and some strains in Group C produced α-haemolysis. A non-haemolytic variant was reported in Group M (Brooke, 1950).

The presence of α-haemolytic strains and minute colony forms in Group L

streptococci emphasizes the similar pattern of variation between the members within each group.

The present work demonstrates the value of grouping in the identification or classification of streptococci irrespective of their haemolytic ability. The β-haemolytic strains were immediately selected for grouping, but those producing α-haemolysis would merely have been classed as *viridans* types had they not been grouped also.

The Group L strains did not appear to be natural pathogens since the sixteen pigs yielding these strains showed no clinical symptoms, although six of them had pneumonic lungs.

SUMMARY.

Sixteen strains of Group L streptococci were found in a collection of fifty-eight strains of streptococci isolated from pigs.

Nine β-haemolytic and two α-haemolytic strains gave typical Group L fermentation reactions, but five other α-haemolytic strains had greater biochemical activities.

One β-haemolytic strain produced typical minute colonies.

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